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CANNABINOID RECEPTOR MESSENGER RNA LEVELS DECREASE IN A SUBSET OF NEURONS OF THE LATERAL STRIATUM, CORTEX AND HIPPOCAMPUS OF TRANSGENIC HUNTINGTON'S DISEASE MICE

E. M. DENOVAN-WRIGHT and H. A. ROBERTSON*

Laboratory of Molecular Neurobiology, Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

Abstract—One of the earliest changes, at the molecular level, that occurs in human Huntington's disease patients is reduction in cannabinoid receptor ligand binding in the substantia nigra pars reticulata compared to neurologically normal controls. The loss of cannabinoid receptor binding is thought to occur early in or prior to the development of Huntington's disease neuropathology. We wish to determine whether cannabinoid receptor messenger RNA levels were altered in a mouse model of Huntington's disease. Transgenic mice hemizygous for the promoter sequence and exon 1 of the human Huntington's disease gene exhibit a progressive neurological phenotype with many of the features of Huntington's disease. This neurological phenotype develops in the absence of neural degeneration making these mice a model system to dissociate changes related to cell dysfunction from changes related to cell loss. We examine the steady-state levels and cellular distribution of the brain-specific cannabinoid receptor messenger RNA by northern blot and *in situ* hybridization. The cannabinoid receptor messenger RNA was expressed throughout the striatum, cortex and hippocampus of wild-type mice. At four and five weeks of age, there was no difference in the distribution of the cannabinoid receptor messenger RNA between the wild-type and transgenic Huntington's disease mice. At six, seven, eight and 10 weeks of age, however, the Huntington's disease mice exhibit reduced levels of cannabinoid receptor messenger RNA in the lateral striatum compared to age-matched controls. The Huntington's disease mice also showed a loss of cannabinoid receptor messenger RNA within a subset of neurons in the cortex and hippocampus. We did not observe any difference in the expression of cannabinoid receptor between the wild-type and Huntington's disease mice throughout Ammon's horn of the hippocampus or in the medial striatum. The decrease in cannabinoid receptor messenger RNA levels preceded the development of the Huntington's disease phenotype and neuronal degeneration and, therefore, these transgenic mice model early cellular changes observed in human patients.

Our results demonstrate that the single copy cannabinoid receptor gene is subjected to cell-specific and time-dependent regulation of the steady-state level of its gene product as a result of the expression of the Huntington's disease gene. As the endogenous cannabinoid receptor agonist, anandimide, has been shown to modulate dopamine neurotransmission within the basal ganglia, the loss of cannabinoid receptors may contribute to the development of motor symptoms or cognitive decline or both seen in Huntington's disease patients. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: basal ganglia, gene expression, *in situ* hybridization, neurodegeneration, northern blot.

The inheritance of a single copy of the huntingtin gene (IT-15) with an expanded CAG repeat within exon I leads to the development of Huntington's disease (HD).[42,49,53] Normally, huntingtin, the protein product of the IT-15 gene, contains a polymorphic, polyglutamine stretch of approximately six to 35 amino acids near the *N* terminus.[49] HD occurs when the number of glutamine-encoding codons is greater than 35.[49] The progression of the disease is affected by the length of the expanded polyglutamine region as there is a decrease in the age of onset and increased rate of progression with increased CAG copy numbers.[50] Huntingtin is ubiquitously expressed throughout the brain and body from early in development throughout adulthood.[7,30,47,51,54] While the function of either the normal or mutated HD form of huntingtin remains to be defined,[45] it is evident that the expression of the HD form of huntingtin eventually leads to progressive but selective neuronal loss. Specifically, the GABA- and enkephalin-containing medium spiny projection neurons of the caudate–putamen die as a result of HD.[35] Patients with minimal cell loss, however, still present with motor symptoms,[26,56] cognitive decline and emotional disturbances[8] suggesting that neuronal dysfunction, and not simply cell loss, contribute to the symptoms of HD.

Changes in the levels of specific receptors have been identified in *post mortem* human HD brains. The dopamine D2 mRNA levels in medium spiny neurons decreases in parallel with the progression of HD pathology.[4,36] Conversely, the amount of D1 mRNA per surviving neuron increases as HD pathology progresses. Additionally, alterations in GABA, muscarinic, cholinergic[31] and glutamatergic[3] receptors have also been reported. Cannabinoid receptor (CB1) ligand binding is also decreased in *post mortem* HD brains.[18,35]

Cannabinoids have recently been shown to be neuromodulators of the dopaminergic system[22] supporting the hypothesis that cannabinoid receptor ligand binding in the CNS contributes to the control of movement.[22] There are two types of cannabinoid receptors CB1 and CB2, which are expressed in the CNS and in the immune system, respectively.[28] CB1 receptors are distributed in the basal ganglia,[24] cerebellum, hippocampus (Ammon's horn and the dentate gyrus), the deeper levels of cortex and the hypothalamus.[1] Cannabinoid receptors are seven-domain membrane spanning proteins

*To whom correspondence should be addressed. Tel.: +1-902-494-8001; fax: +1-902-494-8008.

E-mail address: har1@is.dal.ca (H. A. Robertson).

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CAG, cytosine, adenine, guanine triplet repeat; CB1, cannabinoid receptor; HD, Huntington's disease; IT-5, huntingtin gene; kb, kilobase; nts, nucleotides; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction; OD, optical density; SSC, standard sodium citrate; THC, Δ^9-tetrahydrocannabinol.

coupled to G_i proteins.[1] The endogenous agonist for the cannabinoid receptor is the lipophilic arachidonic acid, ethanolamide (anandamide).[1] Binding of cannabinoid receptor agonists such as the endogenous ligand anandamide or the exogenous agonist Δ^9-tetrahydrocannabinol (THC) effectively lowers cAMP levels within the cell. Anandamide, like THC, produces behavioural effects such as catalepsy, hypomobility and analgesia. The pharmacological actions of anandimide are the same as THC and include inhibition of *N*-type calcium channels and independent mobilization of calcium and arachidonic acid, which may lead to prostaglandin synthesis.[15]

A transgenic mouse model of HD has been created by the integration of a portion of the human huntingtin gene carrying an expanded CAG repeat within the first exon.[27] Both the mRNA and protein encoded by this transgene are ubiquitously expressed in these mice. Although no neuronal degeneration has been observed in the brains of this strain (R6/2) of transgenic mice,[27] these mice display a progressive neurological phenotype that include the development of tremor and abnormal movement that resemble human choreiform movements, a resting tremor and dyskenisia.[9,27] These symptoms suggest that the function of the basal ganglia is affected by the expression of the human exon 1 transgene prior to neuronal cell death.

The objective of this study was to compare the levels of cannabinoid receptor mRNA in wild-type and HD transgenic mice over the period of time that the HD mice develop motor symptoms. Because it had been previously determined that cannabinoid receptor loss was an early cellular change in human HD patients,[18,35] it was of interest to determine at what age the transgenic HD mice have similar cellular changes. Moreover, the fact that HD mice show no signs of neuropathology allowed us to test whether CB1 mRNA loss occurs prior to HD-induced neurodegeneration.

EXPERIMENTAL PROCEDURES

Animals

Animal care was given according to protocols approved by Dalhousie University and the Canadian Council of Animal Care. Every effort was made to ensure the humane and ethical treatment of the animals, and to reduce animal usage. Adult Sprague–Dawley rats (250–300 g; Charles River Laboratories, St. Constant, Quebec, Canada) and wild-type (B6CBAF1) and HD transgenic [B6CBA-TgN(Hdexon1)62Gpb] mice (Jackson Laboratories, Bar Harbor, Maine, U.S.A.) were used in this study. The genotype of the mice was determined by polymerase chain reaction (PCR) amplification of a 100 bp region of the integrated human HD exon 1 transgene using primers corresponding to nucleotides (nts) 3340–3459 (5′-AGGGCTGTCAATCATGCTGG-3′) and nts 3836–3855 (5′-AAACTCACGGTCGGTGCAGC-3′) of clone E4.1 of the human HD gene (Accession number L34020). The PCR conditions used are described in Mangiarini *et al.*[27] DNA was extracted from a tail clip and an ear punch from each mouse used in this study. Both samples were subjected to PCR genotype analysis. For *in situ* hybridization analysis, the animals were anaesthetized with >100 mg/kg sodium pentobarbital, decapitated, the brains removed and stored at −70°C prior to sectioning. For RNA isolation, animals were anaesthetized, decapitated and the striatum and cortex were excised and stored in liquid nitrogen prior to RNA extraction.

In situ *hybridization analysis*

Synthetic oligonucleotide probes were obtained from Sigma Genosys. The CB1 antisense oligonucleotide (5′ ATG TCT CCT TTG ATA TCT TCG TAC TGA ATG TCA TTT G 3′) was complementary to nucleotides 74–110 of the mouse CB1 cDNA (Genbank Accession Number U40709). The control oligonucleotide (5′ CAG CTT TCT CCA CAG GAA CAC AGT AAC AAA GAG 3′) was similar in length and melting temperature to the anti-CB1 probe. Ten pmol of oligonucleotide probe was radio-labelled at the 3′ end with [α-^{33}P]dATP (2000 Ci/mmol; Mandel Scientific) using the reagents and protocol provided in the 3′ end-labelling kit (Amersham Pharmacia Biotech). *In situ* hybridization was performed as described previously.[13] Mouse brain sections were washed twice at 58°C and twice for 30 min in 1× standard saline citrate (SSC), four times for 15 min in 1× SSC, four times for 15 min in 0.5× SSC and twice for 30 min in 0.25× SSC (1× SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0). The slides were rinsed briefly in double distilled H_2O. Rat brain sections were treated following the same protocol except that the washes were carried out at 42°C. The sections were exposed to Kodak MR film for one to three weeks at room temperature. Slides were subsequently immersed in autoradiographic emulsion (Kodak NTB2), allowed to dry and exposed for four weeks at 4°C, prior to developing with Kodak D19 developer and fixer. The sections were counter-stained for Nissl substance using Cresyl Violet (ICN Biochemicals).[37] Densitometric analysis of *in situ* autoradiographs were performed using Molecular Analyst software (BioRad) to determine the optical density (OD) of the radio-label in the lateral and medial striatum and cortex. Measurements were determined on sections derived from three individual animals at each time point. Autoradiographic films were scanned using a BioRad GS-690 imaging Densitometer. Local background was subtracted from each OD value.

Northern blot

RNA was isolated from the striatum and cortex of three 10-week-old wild-type and three 10-week-old HD mice using Trizol (GibcoBRL). The northern blot was prepared by size-fractionating 5 μg of total cellular RNA on a 1% denaturing formaldehyde agarose gel following standard protocols.[44] The CB1 antisense oligonucleotide probe was 3′ end labelled with [α-^{32}P]dATP (3000 Ci/mmol; Amersham) as described above. The northern hybridization conditions have been described previously.[13] A 372 bp cloned PCR product consisting of nucleotides 43–413 of the mouse cyclophilin cDNA (Genbank Accession Number X52803) was radio-labelled using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) and the Ready-to-Go dCTP labelling kit (Amersham Pharmacia Biotech). Following hybridization of the CB1 oligonucleotide probe, the cyclophilin-specific probe was allowed to anneal with the same northern blot to determine the amounts of mRNA present in each lane. Optical densities (OD) of the hybrization signals were determined using Molecular Analyst (BioRad). The OD of the cyclophilin-specific and CB1-specific hybridization was determined for each of three samples derived from individual animals. The local film background was subtracted from each OD value.

Statistical analysis

The OD values for three samples per group for the *in situ* and northern blot analysis were corrected for local background and subjected to Student's *t*-test analysis (one-tailed) with the assumption that the variances were equal. An *F*-test using an alpha level of 0.005 was performed on the same data sets and indicated that in all cases the equal variance assumption was valid.

RESULTS

Localization of cannabinoid receptor mRNA in mouse and rat brain

Oligonucleotides were radio-labelled and used as hybridization probes for *in situ* and northern blot hybridization analysis of the distribution of CB1 mRNA in rats and in wild-type (B6CBAF1) and transgenic HD (B6CBA-Tg(Hdexon1)62Gpb) mouse brains. The antisense oligonucleotide was complementary to nucleotides 74–110 of the mouse brain-specific cannabinoid receptor, CB1. Hybridization reactions using a control probe were run in parallel to determine the level of non-specific probe binding. We initially used these oligonucleotide probes to detect CB1 mRNA in the rat brain as the distribution of the CB1 mRNA in rats has been previously described.[29] The antisense CB1 oligonucleotide had 94.6%

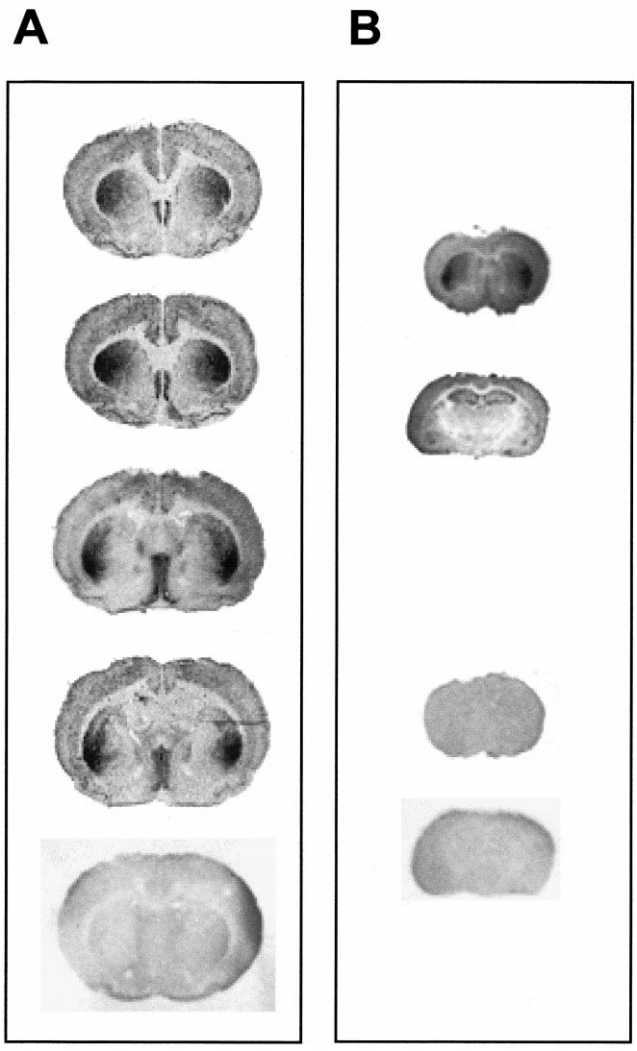

Fig. 1. *In situ* hybridization analysis showing the localization of the CB1 transcript in representative coronal sections of rat brain (A) throughout the rostral–caudal axis of the striatum and in mouse brain (B). The top four rat brain sections (A) were allowed to anneal with the CB1-specific oligonucleotide probe and exposed for seven days. The bottom rat brain section was allowed to anneal with the control oligonucleotide probe and over-exposed for three weeks in order to visualize the outline of various brain regions. The top two and bottom two 10-week-old wild-type mouse brain sections (B) were allowed to anneal with CB1 antisense or control oligonucleotides, respectively. All sections, allowed to anneal with the CB1 antisense or control oligonucleotides, were subjected to the same hybridization and post-hybridization washing conditions as described in the methods.

sequence similarity to the comparable regions of the rat CB1 cDNA sequence (Genbank Accession Number U40395). The mouse and rat cDNA sequences differed at nucleotides 75 (G–A) and 90 (T–C).

Rat brain sections were subjected to *in situ* hybridization analysis using both the CB1 antisense and control oligonucleotide probes (Fig. 1A). CB1-specific hybridization was observed in the striatum, medial septum, descending arm of the band of Broca, the amygdaloid nucleus, Ammon's horn of the hippocampus and cortex (Fig. 1A). CB1-specific hybridization was pronounced in the lateral striatum. In sections derived from rostral brain regions, the hybridization signal was intense in the dorsal lateral striatum. The CB1-specific hybridization signal, however, was concentrated in the ventral lateral striatum in sections derived from caudal brain regions. In addition, dispersed punctate labelling was observed throughout the cerebral cortex (Fig. 1A). Parallel *in situ* hybridization reactions using the control oligonucleotide led to a uniform background signal within the tissue (Fig. 1A). We did not observe any differences in the distribution of the CB1 mRNA-specific signal among the individual animals. These hybridization results using a CB1-specific oligonucleotide probe are similar to those previously published.[29] The CB1 probe, therefore, produced the expected pattern of hybridization in the rat brain and was considered an appropriate probe to detect CB1 in the mouse brain. We anticipated that the pattern of CB1 hybridization would be similar in mouse and rat brains owing to the close phylogenetic relationship between these species and because the striatum appears to be structurally and functionally organized in the same way in these rodents. The CB1 antisense oligonucleotide radiolabelled the same regions in mouse brains (Fig. 1B) as in rat brains (Fig. 1A). The control oligonucleotide did bind non-specifically to the mouse cerebellum, however, this pattern of non-specific probe binding in the cerebellum has been observed with several unrelated probes (data not shown). As such, we did not compare the hybridization of the CB1-specific probe in the cerebellum of wild-type and HD transgenic mice in subsequent experiments.

Northern blot analysis of RNA isolated from the striatum and cortex of 10*-week-old wild-type and Huntington's disease mice*

Northern blot hybridization analysis was done to ensure that the CB1 oligonucleotide probe was annealing specifically to the CB1 mRNA. RNA was extracted from the striatum and cortex of three 10-week-old wild-type and three 10-week-old HD mice. The RNA was size-fractionated by gel electrophoresis on a denaturing agarose gel, transferred to nylon membrane and allowed to hybridize with the radio-labelled CB1 oligonucleotide probe. The oligonucleotide annealed exclusively to a 6.0 kb mRNA in total RNA isolated from the striatum and cortex of each wild-type and HD mouse (Fig. 2). The size of the mRNA that hybridized with the probe corresponded to that reported for CB1 mRNA.[28] To ensure that equivalent amounts of RNA were loaded on each lane of the northern blot, CB1-specific radio-label was removed and the blot was allowed to anneal to a fragment of the mouse cyclophillin cDNA. The uniform hybridization of the cyclophillin probe to the 1.3 kb cyclophilin mRNA present in each lane of the northern blot is shown at the bottom of Fig. 2.

The northern hybridization analysis demonstrated that the relative intensity of CB1 hybridization signal and, therefore, the relative steady-state level of CB1 mRNA was decreased in the 10-week-old HD mouse striatal RNA compared to that of the wild-type mice. CB1 mRNA, however, was still detected in the striatal RNA of the 10-week-old HD mice. In contrast, CB1 mRNA levels did not differ among the wild-type and HD cortical RNA samples. We performed densitometric analysis on the autoradiograms of the northern blot allowed to anneal to both the cyclophilin and CB1-specific probes in order to quantify the apparent decrease in hybridization signal between the striatal RNA of 10-week-old wild-type and HD mice. The OD values for each of three samples derived from wild-type and HD striatum and cortex were corrected for local background radioactivity and subjected to a one-tailed *t*-test. There was no significant difference between the OD of the

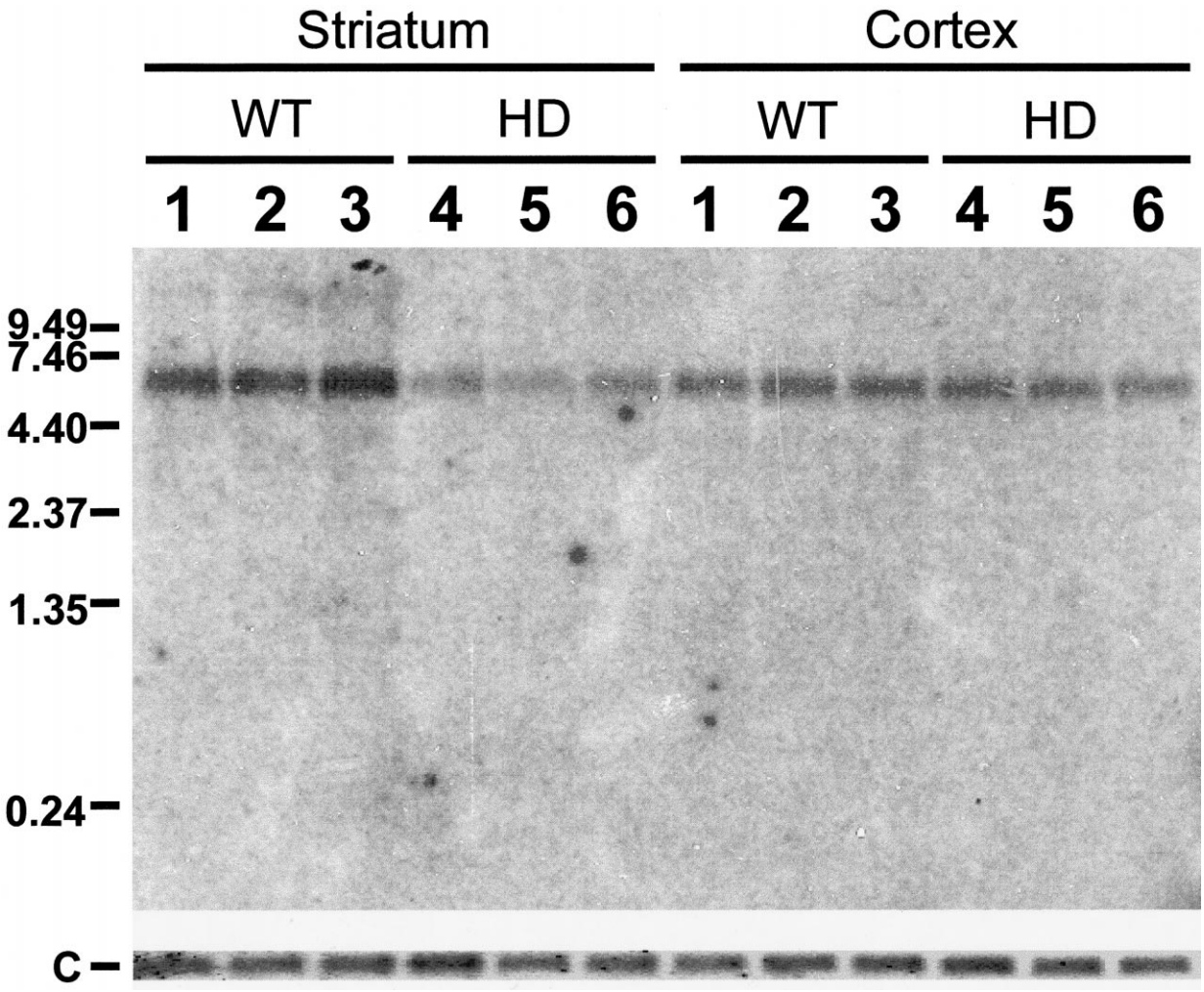

Fig. 2. Northern blot analysis of RNA extracted from the striatum and cortex of 10-week-old wild-type and transgenic Huntington's disease mice. The numbers above the lanes indicate individual animals. Number 1–3 and 4–6 are wild-type and HD, respectively. The RNA derived from the striatum and cortex is indicated. The numbers on the left of the panel indicate the size of the RNA ladder (Gibco, BRL). The bottom panel (indicated by the letter C) shows the hybridization of a cyclophilin probe to the same blot allowed to anneal with the CB1 antisense oligonucleotide demonstrating that comparable amounts of RNA were loaded on to each lane of the gel.

bands resulting from the annealing of the cyclophilin probe ($P = 0.204$). There was also no significant difference between the OD of the CB1-specific labelling of the cortical RNA from the 10-week-old wild-type and 10-week-old HD mice ($P = 0.164$). In contrast, there was a difference between the CB1-specific hybridization signal between the RNA samples derived from the striatum of wild-type and HD 10-week-old mice ($P = 0.002$). The mean CB1-specific OD (minus film background) of the 10-week-old wild-type striatal RNA was 46.6 ± 5.7. The mean CB1-specific OD of the 10-week-old HD striatal RNA was 24.2 ± 3.2. This difference represents a decrease of approximately 50% of the hybridization signal in the HD mice compared to wild-type mice. There was, therefore, a reduction in the steady-state levels of CB1 mRNA specifically in the striatum of 10-week-old HD mice compared to wild-type mice.

In situ *hybridization analysis of the distribution of cannabinoid receptor mRNA throughout the brains of wild-type and Huntington's disease mice over the time*

In situ hybridization analysis using the antisense CB1 oligonucleotide as a probe demonstrated that CB1 mRNA was expressed in wild-type mouse striatal, cortical and hippocampal regions of the brain in the same distribution as that observed in rat brain sections (Fig. 3). The intensity of the hybridization signal in the lateral striatum of the 10-week-old wild-type mice, however, was greater than that observed in the lateral striatum of the 10-week-old HD mice (Fig. 3). By 10 weeks of age, each of the HD mice used in this study had motor symptoms including a resting tremor and dyskenisia as previously described.[27] As the HD-like motor symptoms progressively developed from seven through 10 weeks of age in these transgenic mice, we wished to determine whether the CB1 transcript was ever expressed in the lateral striatum of the HD mice or whether the steady-state levels of the transcript diminished in the striatum in a course that paralleled the development of the motor symptoms. Wild-type and HD mice were sacrificed at four, five, six, seven, eight and 10 weeks of age and their brains were prepared for *in situ* hybridization analysis using the probe complementary to the coding-strand of CB1 (Fig. 3). None of the four-, five-, six- or seven-week-old HD mice had overt motor symptoms. *In situ* hybridization analysis of sections taken throughout the rostral-caudal axis of the striatum showed that CB1 mRNA was expressed in the lateral striatum of four- and five-week-old

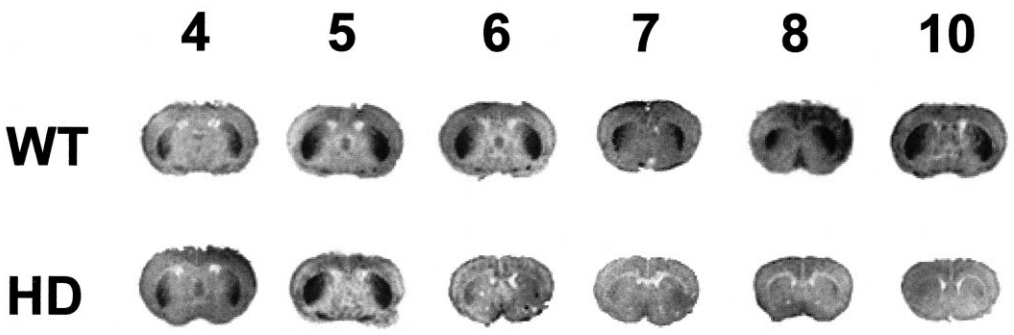

Fig. 3. *In situ* hybridization analysis showing the localization of the CB1 transcript in wild-type and transgenic Huntington's disease mouse brain sections. The numbers above the wild-type (WT) and transgenic HD mouse brain sections indicate the age in weeks of the animals. In sections derived from four- and five-week-old mice, the CB1 mRNA-specific signal has the same distribution in wild-type and HD mice. In seven-, eight- and 10-week-old HD mice, the CB1-specific signal is greatly reduced in the lateral striatum, but not in other brain regions, compared to the age-matched controls.

wild-type and HD mice. The relative hybridization of the probe did not change in four-, five-, six-, seven-, eight- and 10-week-old wild-type mice. The intensity of the hybridization signal was slightly decreased in the lateral striatum of six-week-old HD mice relative to the age-matched wild-type mice. There was no difference in the relative CB1-specific hybridization signal in the lateral striatum among the seven-, eight- and 10-week-old HD mice. The signal, however, appeared to be reduced compared to that observed in age-matched wild-type and to four- and five-week-old HD mouse brain sections. We determined the OD of CB1-specific hybridization in the lateral and medial striatum and cortex of four- and 10-week-old wild-type and HD mice. We found no significant difference between the mean CB1-specific OD in the cortex of four- ($P = 0.207$) and 10- ($P = 0.311$) week-old wild-type or HD mice. Similarly, we found no difference between the medial striatum of the four- ($P = 0.249$) and 10- ($P = 0.472$) week-old wild-type and HD mice. There was no difference between the mean CB1-specific OD in the lateral striatum of wild-type and HD mice ($P = 0.226$). In contrast, there was a significant difference between the mean CB1-specific OD in the lateral striatum of 10-week-old wild-type and HD mice ($P = 0.015$). Together, the *in situ* and northern blot hybridization analysis demonstrated that some, but not all, CB1-expressing cells in the striatum of HD mice have an age-dependent decrease in CB1 mRNA levels.

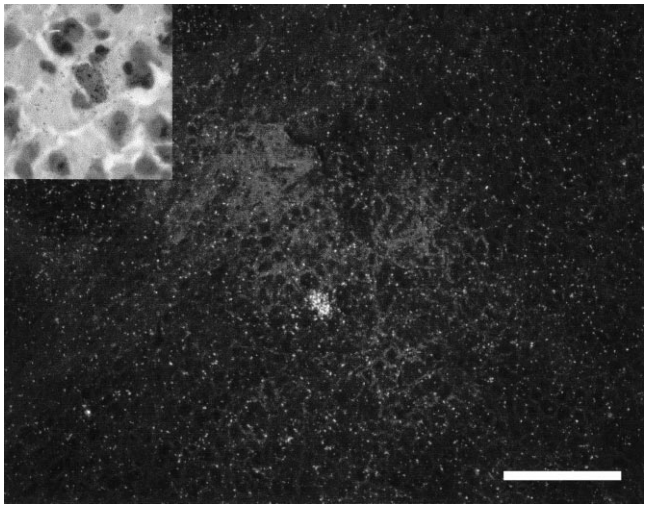

Fig. 4. CB1 mRNA is expressed by isolated neurons in the cortex of wild-type and four- and five-week-old, but not seven- to 10-week-old transgenic Huntington's disease mice. Emulsion autoradiography of cortex from a 10-week-old wild-type mouse showing heavy labelling of an isolated neuron. The section was counter-stained with Cresyl Violet (top left insert) demonstrating that the CB1-specific signal was concentrated over the neuronal cell body. Scale bar = 75 μm.

We did not detect differences between the wild-type and HD mice in the distribution or intensity of the CB1-specific signal hybridization that was observed uniformly throughout the layers of the cortex, medial septum or in Ammon's horn of the hippocampus. However, in addition to the uniform CB1-labelling throughout the cortex and hippocampus, dispersed but intense label was seen throughout the cortex and hippocampus of wild-type mouse (Figs 3 and 4) and rat (Figs 1 and 5) brain sections. These infrequent, dispersed, but intense patches of CB1-specific hybridization were not observed in the seven-, eight- and 10-week-old HD mouse brain sections (Figs 3 and 5).

Following *in situ* hybridization, the wild-type and HD mouse brain sections were directly exposed to autoradiographic emulsion and, after development, viewed under dark-field microscopy. It appeared that the silver grains were uniformly distributed throughout the striatum, cortex and hippocampus in both the wild-type and HD mice. The density of silver grains in the lateral striatum relative to the cortex was in agreement with the autoradiograms of the *in situ* hybridization (data not shown). The intensity of the CB1-specific signal and not the number of cells expressing CB1 were greater in the lateral than in the medial striatum in wild-type and four- and five-week-old HD mice. In the seven-, eight- and 10-week-old HD mouse brain sections, there was uniform label throughout the striatum. At the cellular level, it appeared that the CB1 mRNA-specific signal was localized to medium-sized neurons in the striatum, a distribution that has been described in rats.[29] In addition to the CB1-specific signal dispersed throughout the striatum and cortex, dense clusters of silver grains were observed infrequently throughout the cortex and hippocampus but not the striatum (Fig. 5). The emulsion autoradiography revealed that the distinct punctate labelling was associated with isolated cortical and hippocampal neurons in wild-type and four- and five-week-old but not seven-, eight- or 10-week-old HD mice (Fig. 5). Again, as previously described in rats,[14,29] isolated heavily labelled cells were observed in the pyramidal cell layers, the molecular layer and in the hilar region of the dentate gyrus in wild-type and four- and five-week-old HD mice. The granule cells did not appear to be specifically labelled by the antisense CB1 probe. In agreement with the *in situ* hybridization analysis, we did not observe heavy labelling of individual cells in

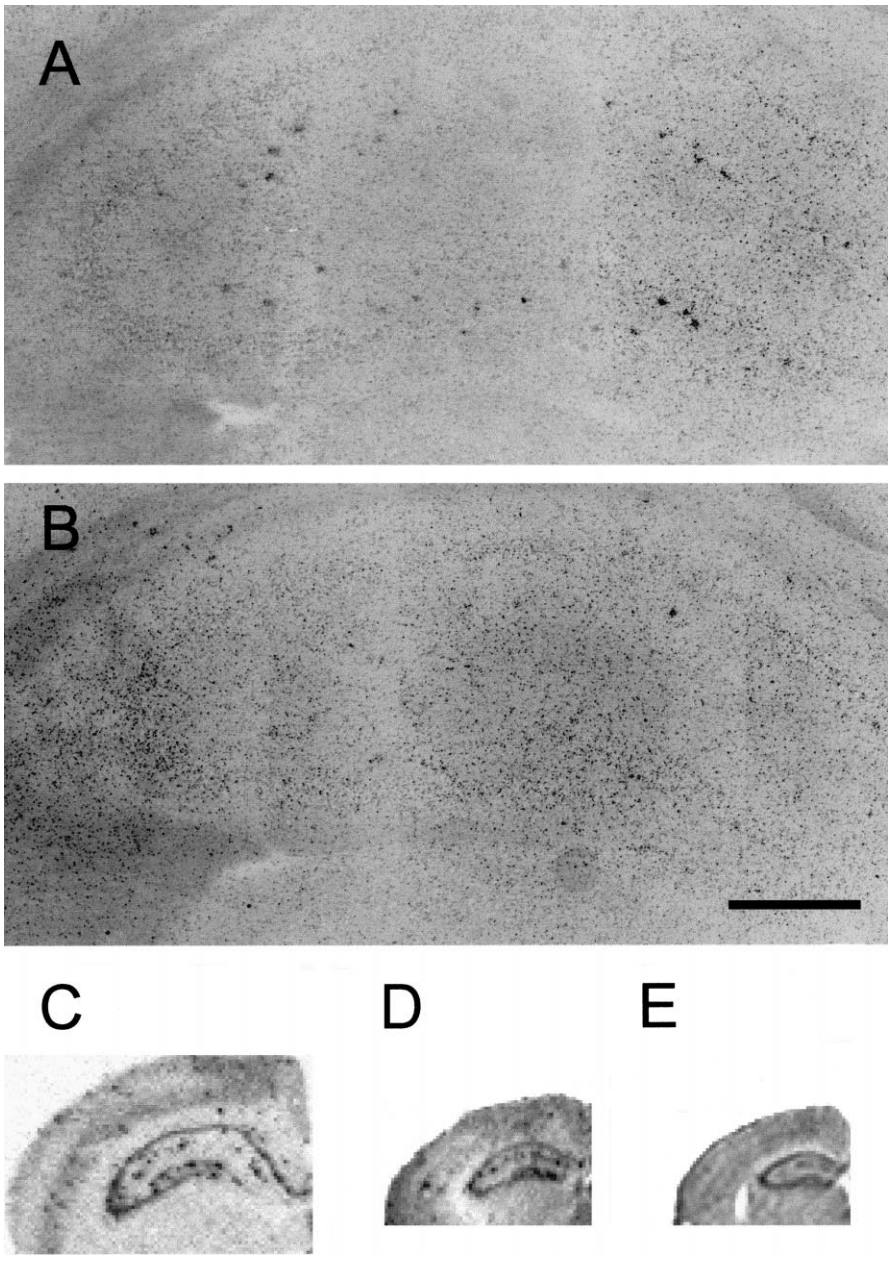

Fig. 5. Hybridization of the CB1 oligonucleotide in the hippocampus. A and B are inverted, composite pictures of hippocampi derived from 10-week-old wild-type (A) and HD (B) transgenic mice following hybridization of the CB1 probe and emulsion autoradiography. Sections derived from rat (C) and 10-week-old wild-type (D) and HD (E) mice allowed to anneal with the CB1 probe and exposed to autoradiography film are shown below. The punctate labelling throughout the cortex and molecular layer of the rat and wild-type mouse brain sections were not observed in the 10-week-old HD mouse brain sections. CB1 labelling throughout CA1, CA2 and CA3 were observed in all brain sections examined. Scale bar = 360 μm (B; also applies to A).

the cortex and hippocampus of seven- to 10-week-old transgenic HD mice by emulsion autoradiography.

DISCUSSION

In situ and northern hybridization analysis demonstrated that the steady-state levels of CB1 mRNA decrease in a specific sub-population of neurons in the lateral striatum, hippocampus and cortex in transgenic HD compared to wild-type mice. CB1 mRNA levels did not differ between four- and five-week-old wild-type and HD mice but, by seven weeks of age, the relative CB1 mRNA levels decreased in specific brain regions in the HD compared to age-matched wild-type mice. Therefore, there is a decrease in CB1 mRNA that occurs before the onset of the motor-related HD-like symptoms in these mice[27] and precedes neural degeneration. These results are in agreement with Glass *et al.*[18] who demonstrated a loss of cannabinoid receptor ligand binding in the substantia nigra pars reticulata of a human HD patient with grade 1,[52] and, therefore, minimal neuropathology.

The steady-state levels of CB1 mRNA did not continually decline in the HD mice overtime. It appeared that the level of CB1 mRNA observed in the wild-type and four- and five-week-old mice decreased from five to seven weeks and then remained constant over the next three weeks in the lateral striatum. In the seven-, eight- and 10-week-old HD mouse brain sections, there was uniform label throughout the

striatum. At the cellular level, the CB1 mRNA-specific signal was localized to medium-sized neurons throughout the striatum in both the wild-type and seven- to 10-week-old HD mice, a distribution that has been described in rats.[29] It appeared that the intensity of the CB1-specific signal decreased in neurons of the lateral striatum in the HD mice to the levels observed in the medial striatum of wild-type and HD mice. Moreover, although we could not detect the intense radio-label associated with isolated neurons of the cortex and hippocampus in seven-, eight- or 10-week-old HD mice, it is was not possible to determine whether these neurons no longer expressed CB1 or if the level of CB1 mRNA in these cells decreased to levels indistinguishable from those of the surrounding neurons. Taken together, these observations suggest that some time after five weeks of age, a change occurs in the HD mice that leads to the reduction, but not complete loss, of CB1 mRNA in specific neurons and that the levels then remain constant at a new, reduced level in these neurons. However, CB1 mRNA levels do not change in other regions of the brain such as in the majority of cells of the cortex, the medial striatum and in Ammon's horn of the hippocampus.

The decrease in CB1 mRNA levels in specific populations of neurons in HD mice contrast with the changes observed in aged rats. Berrendero *et al*.[6] and Romero *et al*.[40] described the age-dependent decrease in CB1 mRNA levels in medial and lateral striatum, all areas of Ammon's horn, the dentate gyrus and brainstem of aged compared to young rats. The isolated, heavily labelled neurons that we, and others, have observed throughout the hippocampus and cortex of rodents are still present in aged rats.[6] In the transgenic HD mice, the expression of the CB1 gene decreased in the lateral striatum but did not change in the medial striatum. Also, we did not observe any difference in the hybridization of the CB1-specific probe to regions of Ammon's horn yet saw the disappearance of label associated with large isolated neurons distributed throughout the pyramidal and molecular layers and hilar region of the hippocampus. Therefore, the regional alterations in CB1 mRNA levels are not the same in aged rats as those observed in transgenic HD mice. These data argue against the possibility that the transgenic HD mouse brain has undergone an increased rate of ageing compared to controls. Moreover, the mechanisms that perturb the expression of CB1 over time in rodents are unlikely to be identical to those of which perturb the expression of CB1 in HD mice.

We have not determined whether the decrease in the steady-state level of the CB1 mRNA specifically in the lateral striatum or in the dispersed cortical and hippocampal cells is a result of a reduction in the rate of transcription or a decrease in the half-life of the CB1 mRNA in these specific cells. If the decrease in the steady-state levels of CB1 mRNA is due to a change in the rate of transcription, it will be of interest to determine whether the HD form of huntingtin is either directly or indirectly interacting with cell-specific transcription factors. It has been postulated, based on protein modelling and yeast screening analysis, that mutant huntingtin may bind to other proteins through polar zippers.[5,23,32–34] Moreover, the factor interaction domain of transcription factors are frequently glutamine-rich leading to the possibility that the mutant form of huntingtin may directly alter transcription or interact with transcriptional factors preventing normal transcription.[48] However, it is also possible that huntingtin with expanded polyglutamine repeats exerts an effect on cell-specific components of the complexes involved in mRNA turnover.[43] Clearly, the promoter of the single copy CB1 gene cannot be directly interacting with the HD form of huntingtin because the levels of CB1 mRNA were unchanged in the majority of brain regions examined. Moreover, as CB1 is encoded by an intron-less gene as a single exon,[28] the differences in the steady-state levels of CB1 mRNA observed in HD mice are not the result of alternate splicing of heteronuclear RNA. The effect of the expression of the HD transgene must be to alter the function of a cell-specific factor(s) involved either in transcription of specific genes or the mRNA stability of particular transcripts.

Within the striatum, the cannabinoid receptors are located on the presynaptic terminals of GABAergic, medium spiny projection neurons.[35] The GABA/enkephalin positive neurons that project to the lateral globus pallidus are lost early in the progression of HD.[35] Subsequently, the GABA/substance P positive efferent neurons that terminate in the internal pallidal region and the substantia nigra are also lost.[18] The nigrostriatal pathway, an important component of extrapyrimidal function, harbours dendrites that contain GABA receptors and extend deep in the substantia nigra to receive contact from these cannabinoid receptor-containing striatonigral efferents.[35,38,39,41] The loss of cannabinoid receptor binding progresses in brain with advanced neuropathology.[35] The imbalance in the distribution of cannabinoid receptors in the lateral and interior regions of the globus pallidus, through cell death or through decreases in the number of functional receptors before cell death, may account for some of the hyperkinetic aspects of the disease.

The endocannabinoid anandimide is a neurotransmitter.[17,19–22,25] Anandimide release is triggered by depolarization and by activation of D2 receptors. Pharmacological blockade of cannabinoid receptors in the striatum potentiates D2-receptor induced hyperactivity. These experiments have led to speculation that negative feedforward or feedback inhibition by anandimide may block the inhibition of adenylate cyclase by D2 receptor activation.[16,22,46] It follows then that stimulation of CB1 receptors may reduce hyperkinetic movements seen in HD.[46] Others have found that application of cannabinoids to the substantia nigra is accompanied by a small decrease in their dopaminergic activity[21] and have speculated that cannabinoids potentiate release of GABA, through GABA-reuptake inhibition, from the striatonigral terminals which inhibits normal dopamine release from these nigrostriatal efferents.[11] For example, muscimol, a GABA agonist, normally causes catalepsy when injected into the globus pallidus of rats but administration of THC by itself does not. However, administration of THC significantly increased the effect of muscimol-induced catalepsy suggesting that the production of catalepsy by THC may depend on its interaction and potentiation of this GABAergic system.[55]

Self (see Ref. 46) suggested that the cannabinoids may be useful to control some of the motor deficits associated with increased dopamine neurotransmission in HD patients. Agonist binding to cannabinoid receptors lowers cAMP concentrations opposing the effect of dopamine binding to D1 receptors[2,18] while cannabinoid receptor blockade potentiates D2 induced hyperactivity.[22] Cannabinoids have been used to treat tremors in multiple sclerosis.[12] The R6/2 transgenic HD mouse,[27] which shows movement abnormalities by eight weeks of age, provides a model system to determine whether

or not cannabinoid agonists or antagonists may be of therapeutic value for the treatment of HD.

In addition to CB1 receptor mRNA decreases documented in this report, the levels of several receptors, as measured by ligand binding or by detection of specific mRNAs, are altered within the brains of transgenic HD mice.[10] Compared to controls, the transgenic mice show decreased NMDA- (*N*-methyl-D-aspartate), AMPA-, kainate-, muscarinic acetyl choline-, D1- and D2-specific ligand binding in the striatum. In contrast, there was no significant difference in striatal ligand binding for $GABA_A$, $GABA_B$, group I metabotropic or dopamine uptake receptors in HD transgenic mice compared to controls. The steady-state level of mGluR1, D1 and D2 but not mGluR2, mGluR3, mGluR5, or NMDA-R1 mRNA, decreased in the striatum of HD transgenic mice compared to wild-type controls. The mRNA levels of mGluR5 and NMDA-R1 did not change in the cortex of the HD mice over time. It seems likely, therefore, that cell-specific factors affect the steady-state levels of specific mRNAs and functional receptors in HD mice. It remains to be determined whether these changes in gene expression are due to the direct or indirect affect of mutant huntingtin. It is unlikely that each of the genes whose expression is altered in HD share a common transcriptional control element that interacts directly with the mutant form of huntingtin because we have demonstrated that the single copy CB1 gene is differentially affected in specific cells over time in HD mice. Moreover, there does not appear to be a simple relationship between the decrease in the steady-state levels of the transcription products of specific genes and their brain-specific patterns of expression. This suggests that the mutant form of HD is not disrupting a single cell-specific transcription factor that specifically controls the expression of these and not other genes. In addition, it remains to be determined whether or not the time-course of the changes in mRNA levels is the same for all genes affected by the HD protein. It seems likely that the mutant HD protein independently affects proteins that control the levels of specific RNAs.

CONCLUSIONS

Our results show that CB1 mRNA levels decrease over time in the lateral striatum and in isolated neurons of the cortex and hippocampus of transgenic HD mice. The decrease in CB1 mRNA levels precedes the development of the HD phenotype and neuronal degeneration and, therefore, these transgenic HD mice model early cellular changes observed in human HD patients. The single copy CB1 gene is subjected to cell-specific and time-dependent regulation of the steady-state level of its gene product as a result of the expression of the HD gene. The alterations seen in the CB1 mRNA levels in the lateral striatum and in isolated neurons of the cortex and hippocampus may contribute to the hyperkinetic phenotype observed in HD mice and patients. Moreover, loss of CB1 mRNA in cells that do not undergo cell death, such as those in the hippocampus, may be involved in cognition and memory decline also seen during the progression of HD. Thus, therapeutic strategies that focus on the retention of these cannabinoid receptors or the augmentation of the function of the remaining CB1 receptors throughout the brain may alleviate some of the symptoms observed in HD.

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