Distribution of Inclusions in Neuronal Nuclei and Dystrophic Neurites in Huntington Disease Brain

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Abstract. Recently, an N-terminal fragment of huntingtin was localized to neuronal intranuclear inclusions (NII), presumed to cause cellular dysfunction, and to inclusions in dystrophic neurites (IDN) in the neostriatum and neocortex of Huntington disease (HD) patients. In the present immunohistochemical study of autopsy brain of 2 juvenile-onset HD patients, 5 HD patients with adult-onset, and 3 controls, NII and IDN as stained with both N-terminal antiserum to huntingtin and ubiquitin antiserum were detected in the HD neostriatum, neocortex, and allocortex, but not in the HD pallidum, cerebellum, and substantia nigra nor in control brain. The frequency of NII in the HD neocortex was highest in the juvenile patients. Within the allocortex, NII and IDN were found in the entorhinal region, subiculum, and pyramidal cell layer of Ammon’s horn. N-terminal huntingtin antiserum also labeled intranuclear granular structures adjacent to the neuronal nuclear membrane in 5 HD patients, one control with idiopathic epilepsy, and one with Alzheimer disease. Our results show that NII formation in HD involves the allocortex in addition to the neostriatum and neocortex. The development of NII in the neocortex and allocortex in HD brain might contribute to the emergence of the cognitive and behavioral symptoms of the disease.

Key Words: Dystrophic neurites; Huntingtin; Huntington disease; Immunohistochemistry; Neuronal intranuclear inclusions; Ubiquitin.

INTRODUCTION

Huntington disease (HD) is an autosomal dominant progressive neurodegenerative brain disorder characterized by motor disturbance, cognitive loss, and behavioral change (1). The neuropathological hallmark of HD is selective loss of medium spiny GABAergic neurons in the neostriatum. Neuronal loss has also been reported in (among others) the neocortex, pallidum, cerebellum, allocortex, and substantia nigra (2, 3).

The mutation underlying HD is an expanded CAG/polyglutamine repeat in the first exon of a gene on chromosome 4 encoding a ~348 kDa protein of unknown function, called huntingtin (4). The repeat expansion on chromosomes of affected individuals ranges from 36 to 180 copies (5, 6). The repeat size correlates inversely with age of onset (7–9).

Huntingtin is widely expressed, with highest levels of expression in brain, where it is predominantly neuronal (10–12). Within neurons, huntingtin is present in the cytoplasmic compartment and distributed in the perikaryon, axon, dendrites, and nerve terminals (13–17). In addition, huntingtin has been observed within the nucleus of neurons in human and monkey brain (13, 18) and in the nucleus of various cultured mammalian cells (19, 20).

Both wild-type and mutant huntingtin are expressed in HD brain (21, 22).

HD belongs to a growing number of CAG/polyglutamine disorders including several forms of spinocerebellar ataxia (SCA) (23). In HD-transgenic mice and in patients with HD, neuronal intranuclear inclusions (NII) reactive to N-terminal antiserum to huntingtin have recently been identified (24, 25). NII were found in the HD neostriatum and neocortex, both also harboring inclusions in dystrophic neurites (IDN), and were most dense in the same regions in the transgenic mice. NII, containing the disease protein with an expanded polyglutamine repeat, have also been demonstrated in the affected brain areas in patients with SCA-3 and SCA-1 and in Purkinje cells in SCA-1 mice (26, 27). In SCA-7 brain, NII were detected with an antibody recognizing expanded polyglutamine domains (28). The inclusions also contain ubiquitin. In both mouse models, a neurological phenotype develops only after the appearance of NII and in the absence of or long before obvious neuronal death, indicating that NII may cause neuronal dysfunction (23, 24, 27).

This immunohistochemical study evaluates the possibility that nerve cells in brain regions apart from those preferentially affected in HD, yet showing loss of neurons, might be affected by NII and IDN formation. Autopsy brain tissue was analyzed with antibodies to the N-terminus and an internal region of huntingtin as well as to ubiquitin, and with human serum harboring anti-neuronal autoantibodies as a pan-neuronal marker (29, 30).

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TABLE 1

Data of Huntington Disease (HD) Patients and Controls

<table>
<thead>
<tr>
<th>Pat. no.</th>
<th>Sex/Age onset (yr)</th>
<th>Disease duration (D) (yr)</th>
<th>CAG repeats (CAG)n</th>
<th>Brain weight (g)</th>
<th>Pm delay (hr)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gr</td>
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<td>HD patients</td>
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<tr>
<td>1. f/I1</td>
<td>6 5 22-84</td>
<td>1,240 4</td>
<td>21</td>
<td></td>
<td></td>
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<tr>
<td>2. f/I9</td>
<td>7 12 17-86</td>
<td>860 4</td>
<td>5</td>
<td></td>
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<tr>
<td>3. f/I41</td>
<td>21 20 16-46</td>
<td>1,150 3</td>
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<td></td>
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<td>840 4</td>
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<tr>
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<td></td>
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<tr>
<td>6. f/I63</td>
<td>49 14 21-45</td>
<td>nd 3</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. m/67</td>
<td>48 19 16-41</td>
<td>1,125 3</td>
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</tr>
<tr>
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<td>*lung carcinoma</td>
<td>1,380 4</td>
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<td>*alzheimer disease</td>
<td>1,270 8</td>
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</table>

1 Patients: mean = 46 ± 21 yrs. Controls (nos. 8–11): mean = 48 ± 19 yrs.
2 Patients: mean = 1,075 ± 165 gr. Controls (nos. 9–11): mean = 1,356 ± 17 gr; D = disease duration; (CAG)n = number of CAG repeats in the HD alleles; Gr = grade according to the classification of Vonsattel et al (31); Pm = postmortem; Nd = not determined; * = clinical diagnosis.
3 Cause of death: status epilepticus.
4 The brain weight reflects massive edema.

MATERIALS AND METHODS

Patients and Controls

Brain tissue (striatum [caudate nucleus, putamen, pallidum], neocortex, allocortex, substantia nigra, and cerebellum) was obtained at autopsy from 2 juvenile and 5 adult HD patients and 5 controls. The clinical diagnosis of HD was confirmed by neuropathological evaluation and determination of the CAG repeat length in all patients. HD severity was graded according to the classification of Vonsattel et al (31). The data are summarized in Table 1.

Antibodies

Antiserum 7 was raised in rabbit to synthetic peptides corresponding to the first 19 amino acids of huntingtin and affinity purified using an affinity column according to the procedure supplied by the manufacturer (Pierce, Rockford, IL). Briefly, a total of 2 mg of peptide was coupled to column matrix and subsequently the sera were applied. After washing the column, bound antibodies were eluted using 0.1 M Glycine, pH 2.5, and the collected fractions were immediately neutralized with 1 M Tris, pH 9.5. The specificity of the purified antibodies was examined by Western blot experiments, performed as described by De Rooyij et al (20). The preparation, characterization, and specificity of rabbit antiserum 93 against amino acids 1929–2421 of huntingtin have been previously described (20). Rabbit antiserum against ubiquitin was obtained from DAKO (Denmark). Human anti-Hu (or anti-neuronal nuclear antibody type 1, ANNA-1) serum was obtained from a patient with a subacute sensory neuropathy without known tumor. This serum contained a high titer of antibodies against Hu antigens, a family of neuron-specific proteins (29, 30).

Immunohistochemistry

Immunolabeling was performed on 5-μm-thick sections cut from 10% formalin-fixed and paraffin-embedded tissue blocks. Prior to pretreatment by boiling in citrate buffer (pH 6.0) for 20 minutes (min) all sections were preincubated in 0.3% H2O2 to block endogenous peroxidase. For staining with anti-Hu serum an additional preincubation was performed with normal goat serum (1:10). The primary antiserum-antiserum 7 (1:10), antiserum 93 (1:100), and ubiquitin antiserum (1:800)- and anti-Hu serum (1:200) were incubated on the sections overnight at room temperature. Next, the sections were incubated with biotinylated swine anti-rabbit immunoglobulin (Dako) for 30 min for the antisera or with biotinylated goat anti-human immunoglobulin (Vector) for 60 min for anti-Hu serum, followed by peroxidase-conjugated streptavidin (Dako) for 30 min. Peroxidase activity was visualized using 3,3′-diaminobenzidine as the chromogen. Controls were obtained by omission of the primary antisera.

Quantitation of NII

Hu immunostaining (Fig. 1) allowed a more accurate determination of the number of neurons (particularly smaller neurons) in a given area than in sections stained with antiserum 7. Therefore, the frequency of NII was determined by counting the number of nucleated neurons and the number of neurons with NII in 3 corresponding fields spanning the entire thickness of the neocortical gray matter in immediately adjacent sections stained with anti-Hu serum or antiserum 7, respectively, at ×400 magnification and using a 10-mm² ocular grid.

RESULTS

Neuronal Intraneuronal Inclusions

Antiserum 7 against N-terminal huntingtin immunolabeled NII in the neostriatum, neocortex, and allocortex of all HD patients in whom these areas were investigated (Table 2). The NII were also ubiquitin-positive. Antiserum 7-positive NII were not detected in the HD pallidum, cerebellum, and substantia nigra. The nuclear inclusions were round-, oval-, or rod-shaped. Their size varied from smaller to considerably larger than the nucleolus (Fig. 2). Occasionally, a nucleus contained 2 NII. NII were not detected with antiserum 93 raised against an internal region of huntingtin. The specificity of antiserum 7 is shown in Western blot experiments by the detection of one major band, i.e. wild-type huntingtin, in extracts prepared from human normal fibroblast VH10 cells and the detection of 2 bands, i.e. wild-type huntingtin and mutated huntingtin, in extracts prepared from patient-derived lymphoblasts (Fig. 3). Evidently as a result of the severity of the neuronal loss in this region, NII were infrequently found in the neostriatum.

Although NII were present in neurons of all layers of the neocortex, they were more prevalent in layers V, VI,
Fig. 1. HD patient 4. Strip of neocortex (P, pial surface; WM, white matter) showing neurons immunostained with anti-Hu serum, ×40.

Fig. 2. HD patient 3. Neocortical neuron with a large intranuclear inclusion and an intranuclear granular structure adjacent to the nuclear membrane. Note the immunostaining of the neuronal cytoplasm. Antiserum 7 immunostaining, ×600.

and III in the adult-onset patients. In both juvenile patients, many neurons with NII were also seen in layer II. For each patient, the percentage of neocortical neurons containing antiserum 7-positive NII is presented in Table 2.

Within the allocortex, NII were detected in neurons of the entorhinal region and subiculum, and in pyramidal cells of Ammon’s horn (Fig. 4), but not in dentate granule cells. NII were conspicuously frequent in the subiculum in patient 3 and even more strikingly in patient 1, where the inclusions were locally present in more than 75% of neuronal nuclei. Within Ammon’s horn, NII were limited to sector CA1 or dispersed throughout CA1–CA3, either evenly or with local enrichment (to NII in 60% of neurons) in one or 2 sectors (Fig. 5). Only in the juvenile-onset patients were rare NII present in CA4. NII were

### TABLE 2

<table>
<thead>
<tr>
<th>Pat. no.</th>
<th>Nctx u/h</th>
<th>%</th>
<th>CN u/h</th>
<th>P u/h</th>
<th>GP u/h</th>
<th>ECtx u/h</th>
<th>S u/h</th>
<th>CA u/h</th>
<th>SN u/h</th>
<th>Ce u/h</th>
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<td>-/-</td>
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<tr>
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<td>na</td>
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</tr>
<tr>
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<td>+/+</td>
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</tr>
<tr>
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<td>-/-</td>
</tr>
<tr>
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<td>2.6</td>
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<td>+/+</td>
<td>-/-</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>-/-</td>
</tr>
</tbody>
</table>

Nctx = neocortex; % = percentage of neocortical neurons with antiserum 7-immunoreactive NII; CN = caudate nucleus; P = putamen; GP = globus pallidus; ECtx = entorhinal cortex; S = subiculum; CA = cornu Ammonis; SN = substantia nigra; Ce = cerebellum; + = present; - = absent; na = not available.

not observed in the cerebellar Purkinje and granule cells, nor in the dentate nucleus, which was available for examination in HD patient 2.

NII showing immunoreactivity to antiserum 7 were absent in both pigmented and nonpigmented neurons of the substantia nigra. In 5 of 7 HD patients, however, a variable number of pigmented neurons contained prominent, often multiple, ubiquitin-immunoreactive NII (Fig. 6). These so-called Marinesco bodies are thought to reflect an involutional change (32–34). They were also found in neurons of the substantia nigra in 2 controls. Otherwise, NII were absent in the control brains.

Inclusions in Dystrophic Neurites

Antiserum 7 labeled round or ovoid IDN in the HD neocortex and less frequently in the neostriatum and allocortex (Fig. 7a, c). IDN were ubiquitin-positive and did not stain with antiserum 93. Neocortical IDN were more prevalent in the patients with adult-onset than in the juvenile patients and were predominantly localized in layers V and VI. Within the allocortex, IDN were mostly found in CA1–CA3 of Ammon’s horn (Fig. 7b, c). They were absent in the HD pallidum, cerebellum, and substantia nigra. IDN were not found in control brain.

Additional Immunohistochemical Findings with Antiserum 7

In control and HD brain, the cytoplasm of neuronal cell bodies and the emerging axons and dendrites showed variably intense immunostaining with antiserum 7 (Figs. 2, 7b, 8a, c). The immunostaining was punctate in its distribution within the cytoplasm. Antiserum 7 also labeled individual fibers traversing the gray matter (Fig. 7a) and axons in myelinated fiber tracts.

Within neuronal nuclei, antiserum 7 immunostained granular structures lying apposed to the nuclear membrane (Figs. 2, 8b, c). These structures did not show immunoreactivity with ubiquitin antiserum nor with antiserum 93 raised against an internal region of huntingtin. They were seen in neocortical neurons in 5 HD patients, one control with idiopathic epilepsy, one with Alzheimer disease (AD), as well as in pyramidal neurons of Ammon’s horn in one HD patient.

DISCUSSION

The main result of this study is the localization of NII and IDN staining with N-terminal antiserum to huntingtin and with ubiquitin antiserum to the allocortex of HD brain. Our study confirms the presence of these pathological structures in the HD neostriatum and neocortex (25, 35, 36). They were not observed in the HD substantia nigra or, in agreement with a previous report (25), in the pallidum and cerebellum.

Within the neocortex, the frequency of neurons with NII appeared to be higher in the juvenile than in the adult HD patients. Both juvenile patients had over 80 CAG repeats. In the adult patients, the number of repeats varied between 40 and 50 units. These results are in agreement with recent studies demonstrating a correlation between increasing CAG repeat length and increasing density of NII in HD (23, 25). The comparatively lower frequencies observed in the present study may relate to technical factors and the different method used for the quantitation of neurons.

NII formation affects the neostriatum and neocortex in HD brain (25, this study). However, our study shows widespread involvement also of the allocortex with NII present in the entorhinal cortex, subiculum, and all sectors of Ammon’s horn. The clinical symptoms in HD include cognitive decline and behavioral changes (1). In view of the findings in HD mice suggesting that NII cause cellular dysfunction (24), one may speculate that the formation of nuclear inclusions in neocortical and allocortical neurons in HD contributes to the emergence of these symptoms.

In the HD neocortex, neuron loss affects layer V and to a lesser extent layers V and III with preferential involvement of pyramidal cells (37–40). In this study, the distribution of neocortical NII showed a similar laminar pattern with, in addition, conspicuous involvement of layer II neurons in the juvenile patients. Within the allocortex, neuron loss has been reported in the entorhinal region, the subiculum, and sector CA1 of Ammon’s horn (41, 42). Our finding of NII in all 4 sectors of Ammon’s horn may indicate a more widespread involvement of this structure in HD pathology than suggested by the localized
Fig. 4. HD patient 5. Large inclusion in the nucleus of a pyramidal neuron in Ammon's horn. Ubiquitin immunostaining, ×400.
Fig. 5. HD patient 3. Neurons with intranuclear inclusions in sector CA2 of Ammon's horn. Ubiquitin immunostaining, ×400.
Fig. 6. HD patient 5. Marinesco body in a pigmented neuron of the substantia nigra. Ubiquitin immunostaining, ×400.

loss of neurons. Decrease of neurons has also been described, though not consistently, in the HD pallidum and substantia nigra (2, 43–45). In particular in juvenile patients, the cerebellum sustains loss of Purkinje and granule cells and neurons of the dentate nucleus (2, 43, 46). Thus, neuronal loss may be a feature in all HD brain regions presently investigated. However, the presence of NII appears to be limited to neurons in the neostriatum, neocortex, and allocortex (25, this study). If, indeed, intranuclear inclusions ultimately cause neuronal death (23), an issue also of interest with regard to Marinesco bodies (see above), NII formation may account for the neuronal loss observed in these regions. Consequently, nerve cell loss in regions of HD brain apparently devoid
of NII must depend on other factors. However, whereas loss of neostriatal efferents may account for the decrease of pallidal neurons, the pattern of neuronal loss in the substantia nigra appears not to correspond to the local distribution of the striatonigral fibers known to degenerate in HD (45). Likewise, the decrease of cerebellar Purkinje and granule cells in juvenile HD is not likely to be the result of transneuronal degeneration. Clearly, further studies are needed to resolve the factors responsible for the widespread neuronal death in HD brain.

Among the presently unknown factors governing the differential vulnerability of neurons to NII formation, proteins interacting with huntingtin, cell type-specificity of proteases involved in the N-terminal cleavage of huntingtin, and the level of expression of huntingtin may play a role (23, 24). The latter shows regional variation and heterogeneity within particular regions including the neostriatum (15, 47, 48). Interestingly, huntingtin immunoreactivity seems to be particularly associated with neostriatal neurons selectively degenerating in HD (47).
In agreement with reports on the cytoplasmic localization of huntingtin (13–17), antiserum 7 stained the neuronal cytoplasm in control and HD brain. In addition, this antiserum labeled nonubiquitinated granular structures in neuronal nuclei in HD patients, one control with AD, and one dying from status epilepticus. This finding may be of interest, since huntingtin can be cleaved in its N-terminus by apopain, an apoptosis associated protease (49), whereas apoptosis has been implicated in neuronal death in all 3 conditions mentioned above (50–55). However, additional studies are required to determine the specificity and significance of these nuclear structures.

In conclusion, N-terminal huntingtin was immunolocalized 1) to the cytoplasm and to intranuclear structures of unknown significance in neurons of both control and HD brain and 2) to ubiquitinated inclusions in neuronal nuclei and neurites in juvenile and adult HD brain. The inclusions appeared to be widely distributed in the allocortex in addition to their already known neostriatal and neocortical localization. The development of NII in the
allocortex and neocortex might contribute to the emergence of cognitive and behavioral dysfunction in HD.

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