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NEURONAL INTRANUCLEAR INCLUSIONS AND NEUROPIL AGGREGATES IN *Hdh*$^{CAG(150)}$ KNOCKIN MICE

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Abstract—We studied the development of neuronal intranuclear inclusions (NIIs), neuropil aggregates (NAs), and expression of expanded repeat polyglutamine protein in the *Hdh*$^{CAG(150)}$ knockin mouse model of Huntington's disease (HD). Diffuse nuclear localization of huntingtin protein (htt) was noted initially within striatal neurons at approximately 28 weeks, followed by the development of striatal htt immunoreactive NIIs by approximately 40 weeks. Striatal NIIs were observed initially in clusters within the matrix compartment but subsequently became diffusely distributed throughout the striatum. In the oldest animals (107 weeks), NIIs were enlarged and diffuse nuclear htt immunoreactivity reduced. Expression of ubiquitin immunoreactive NIIs paralleled but lagged behind the expression of htt immunoreactive NIIs. Abundant NIIs were found by approximately 75 weeks in layers 3 and 4 of somatosensory cortex and in layer 2 of piriform cortex. In the oldest animals, greater than 100 weeks, some NIIs were found in many brain regions. NAs were found mainly within the globus pallidus and substantia nigra, perhaps reflecting expression in striatal terminals. Cyclic AMP response element binding protein (CBP) was not localized to NIIs, arguing against gross sequestration of this transcriptionally active protein. Comparison of the relative levels of a common polyglutamine epitope in *Hdh*$^{CAG(150)}$ knockin and *hprt*$^{CAG(146)}$ knockin mice shows greater expression of the polyglutamine epitope in the phenotypically less aggressive *Hdh*$^{CAG(150)}$ knockin line. *Hdh*$^{CAG(150)}$ knockin mice may be a model of early pathologic changes in HD.

Key words: Huntington disease, polyglutamine, neurodegeneration, ataxia, striatum.

Huntington disease (HD) is the most common of the expanded CAG/glutamine (polyQ) family of triplet repeat disorders (Taylor et al., 2002). HD is distinguished by prominent striatal atrophy though more widespread pathology occurs with disease progression (Robitaille et al., 1997). Gene expression does not explain the regionally restricted pattern of pathology, as *huntingtin* is expressed widely (Strong et al., 1993; Sharp et al., 1993). In common with other polyQ diseases, HD exhibits neuronal intranuclear inclusions (NIIs) and neuropil aggregates (NAs) containing polyQ fragments and other proteins (Tellez-Nagel et al., 1974; Davies et al., 1997; DiFiglia et al., 1997; Paulson et al., 1997a, b; Becher et al., 1998).

Murine genetic models are invaluable in exploring the pathogenesis of polyQ disorders and in preclinical studies of potential interventions (Zoghbi and Botas, 2002; Menalled and Chesselet, 2002; Beal and Ferrante, 2004). NIIs, for example, were recognized initially in a murine transgenic model of HD (Davies et al., 1997). A variety of transgenic and 'knockin' (KI) models of HD have been generated (Mangiarini et al., 1996; Reddy et al., 1998; Hodgson et al., 1999; Schilling et al., 1999; Shelbourne et al., 1999; Wheeler et al., 2000, 2002; Lin et al., 2001; Menalled at al., 2003). An ideal murine genetic model would possess the same genetic defect as HD, mimic the behavioral and pathological features of HD, have easily measured behavioral and pathologic changes, and exhibit these characteristics in a time scale of weeks to a few months. No model yet developed has all these attributes (reviewed by Beal and Ferrante, 2004). Transgenic models based on expression of exon 1 of human *huntingtin* or full length human *huntingtin* constructs containing pathological expanded CAG domains have been very useful. Some of these lines, notably the widely used R6/2 line developed by Bates' group, have an early onset and rapidly fatal behavioral phenotype. Use of only a small portion of the HD gene, transgene integration site effects, and unnatural promoters that boost expression to supraphysiologic levels may distort the pathophysiology of expanded polyQ effects in these models. In R6/2 mice, expression of NIIs and NAs is widespread and does not match the known distribution of pathology in HD. KI models in which expanded CAG repeats have been inserted into the very similar murine *huntingtin* homologue (*Hdh*) have been constructed. These models exhibit milder behavioral alterations than transgenic models but more specific expression of NIIs within the striatum. Studies in a combination of models may be necessary to establish certainty about mechanisms of pathogenesis and for rigorous preclinical studies. The regional distribution of NIIs and NAs has been studied as a function of age systematically in two models, the R6/2 line and a KI line expressing 140 CAG repeats (Morton et

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Abbreviations: CBP, cyclic AMP response element binding protein; DAB, diaminobenzidine; DARPP-32, dopamine and cyclic AMP regulated phosphoprotein-23 kDa; HD, Huntington disease; htt, Huntingtin protein; KI, knockin; NA, neuropil aggregate; NIIs, neuronal intranuclear inclusions; PB, phosphate buffer; polyQ, polyglutamine; RT, room temperature; SCA, spinocerebellar ataxia; TBST, Tris-buffered saline with 0.05% Tween-20.

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al., 2000; Menalled et al., 2003). We described previously a KI line expressing approximately 150 repeats with a late onset behavioral phenotype and selective expression of NIIs in the striatum ($Hdh^{CAG(150)}$ KIs; Lin et al., 2001). We now describe the regional expression of NIIs and NAs as a function of age, explore their modification with ubiquitin, and their association with CBP in this line. We also compare levels of polyQ epitope in this line with those found in another KI line with different rate of NII accumulation

EXPERIMENTAL PROCEDURES

Animals

A total of 81 mice, ages 18–107 weeks, were used in this study. All animals were genotyped by polymerase chain reaction amplification of tail DNA according to the method of Lin et al. (2001).

Immunohistochemical analysis and quantification of NIIs

All efforts were made to minimize the number of animals used and their suffering. All experiments conformed to named local and international guidelines on the ethical use of animals. Homozygous and heterozygous $Hdh^{CAG(150)}$ KI mice were deeply anesthetized with Avertin (Aldrich, St. Louis, MO, USA; 0.4 mg/g) and intracardially perfused with 0.1 M phosphate buffer (PB, pH 7.4) followed by 4% paraformaldehyde in PB. Brains were dissected and immersed overnight in cold fixative, cryoprotected in graded sucrose solutions (up to 20% in PB) and frozen in −70 °C isopentane. Frozen sections were cut at 40 μm in the coronal plane using a sliding microtome. All antibody labeling was performed on free-floating sections, with three 15 min washes in PB between each step. For immunoperoxidase staining, primary and secondary antibodies were diluted in PB, 0.3% Triton-X 100, and 1.5% blocking serum. Primary antibodies were localized using species-specific Vector ABC Elite kits (Vector Laboratories, Burlingame, CA, USA) and the reaction product was developed by incubating the sections in Stable DAB (diaminobenzidine; Invitrogen, Carlsbad, CA, USA). Neuron cell bodies were counterstained with Methyl Green solution (Vector Laboratories) for 5 min at 60 °C, rinsed, dipped in acetone (0.05%), dehydrated and coverslipped. Primary antibodies used in these studies were directed against the N-terminal of huntingtin (1:250; catalog no. SC-8767; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ubiquitin (1:250; catalog no. Z0485; Dako, Denmark), cyclic AMP response element binding protein (CBP; catalog no. SC-369; 1:400; Santa Cruz Biotechnology) and dopamine and cyclic AMP regulated phosphoprotein-23 kDa (DARPP-32; 1:10,000, gift from Dr. Paul Greengard). For light microscopic double-label immunofluorescence studies, sections were incubated overnight in a cocktail of anti-calbindin mouse monoclonal (1:5000; catalog no. 300; Swant, Bellinzona, Switzerland) and anti-huntingtin (1:250) antibodies. Primary and secondary antibodies were diluted in PB, 0.3% Triton X-100, and 10% normal donkey serum. The sections were rinsed and incubated for 1 h in a mixture of FITC-labeled donkey anti-goat (1:150) and SP-biotin-labeled donkey anti-mouse (1:600; Jackson Laboratories, West Grove, PA, USA) antibodies. After rinses, the sections were incubated in streptavidin conjugated with Red-X fluorochrome (1:100 in PB; Jackson Laboratories). Studies utilizing confocal microscopy were conducted using tissue incubated in the same primary antibody combination as above followed by labeling with secondary antibodies conjugated to fluorescent Alexa dyes (Alexa 488 and 594, both at 1:800 in PB; Molecular Probes Eugene, OR, USA). For fluorescent counterstaining, tissue was rinsed in PB and incubated in bisbenzimide (1:1000 in PB) for 5 min at RT. NII quantification was performed on both ubiquitin- and huntingtin-ABC/DAB-stained sections. Neurons were counted on the same sections with Methyl Green counterstaining. Only neurons with evident nuclei were counted. NIIs were counted on a Nikon MicroPhot within a 0.0125 mm^2 area using a grid ocular. Five sections per region per animal were counted and a mean established. For striatum, sections were approximately evenly spaced throughout the striatum, though most rostral and caudal striatum were not included. Data are expressed as the percentage of neurons containing NIIs per 0.0125 mm^2 tissue. The percentage of NAs was established by counting all morphologically similar immunostained structures (NIIs+NAs) within the grid in Methyl Green-counterstained tissue. NIIs were counted as structures associated with a counterstained nucleus, while NAs were not associated with a counterstained nucleus. Photographs of double-labeled tissue were taken on an Olympus FV-500 confocal microscope.

Western blotting

Twelve week old $Hdh^{CAG(150)}$ KI and $Hprt^{(CAG)146}$ KI mice were rendered unconscious by CO_2 asphyxiation and decapitated. The brains were quickly removed, rinsed in ice cold 0.32 M sucrose, and placed on 0.32 M sucrose wetted 3MM paper in a Petri dish on ice. The cortex and striatum were dissected out. These brain regions were frozen quickly in 1.5 ml centrifuge tubes prechilled on dry ice and stored at −80 °C. Tissue was Dounce homogenized in lysis buffer with protease, phosphatase and caspase 3 inhibitors; 400 μl lysis buffer was used for cortex and 100 μl for striatum. The protein concentration of total tissue lysates was assayed using the BCA protein assay kit (Pierce, Rockford, IL, USA). Twenty micrograms of each sample was loaded on a 6% PAGE gel and transferred to a nitrocellulose membrane for 1.5 h at 100 V. Blots were blocked with Tris-buffered saline with 0.05% Tween-20 (TBST) and 5% milk powder for 30 min at room temperature (RT). The blots were probed with the monoclonal anti-polyQ antibody 1C2 (Chemicon, Temecula, CA, USA; Trottier et al., 1995) at 1:10,000 overnight at 4 °C, washed with TBST 3×10 min at RT, probed with HRP-conjugated secondary for 1 h at RT, washed with TBST 3×5 min, 3×10 min at RT and developed. Blots were probed with a monoclonal anti-α-tubulin antibody (1:30,000; cell line B-5-1-2; Sigma) for 1 h at RT, washed with TBST 3×15 min at RT, probed with HRP-conjugated secondary for 1 h at RT, washed with TBST 3×15 min at RT. The blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

Time course and expression of intranuclear htt immunoreactivity and NIIs

Within the striatum, intranuclear htt immunoreactivity was first documented at 27–29 weeks with diffuse nuclear staining in KI animals. Diffuse nuclear staining was seen in neurons throughout the striatum (Fig. 1), but was not apparent in other regions. At approximately 40 weeks, diffuse nuclear staining persisted and htt immunoreactive NIIs were seen clustered in a small percentage of striatal neurons (Figs. 1, 2; Table 1). Immunofluorescent double-labeled htt-stained tissue with bisbenzimide nuclear stain confirmed the intranuclear location of the NIIs (Fig. 2). NIIs appeared slightly earlier (37 weeks) in homozygous animals as compared with heterozygotes (40–42 weeks). By 70 weeks, htt immunoreactive NIIs were distributed widely throughout the striatum with persistent diffuse nuclear staining. NIIs were seen in approximately 40% and 65% of