Neurons Lacking Huntingtin Differentially Colonize Brain and Survive in Chimeric Mice

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Huntington’s disease (HD) is an dominant hereditary neurodegenerative disorder characterized by progressive cognitive decline and motor dysfunction (Bruyn and Went, 1986; Wilson et al., 1987; Albin and Tagle, 1995). The major site of neuron loss in HD is the striatal part of the basal ganglia, and it is this loss that accounts for the progressive movement disorder (Vonsattel et al., 1985; De La Monte et al., 1988; Hedreen et al., 1991; Storey et al., 1992). The gene and the specific mutation responsible for HD have been known for several years (Huntington’s Disease Collaborative Research Group, 1993). The gene product, huntingtin, is of unknown function, although several lines of evidence suggest that it is involved in vesicular trafficking (DiFiglia et al., 1995; Sharp et al., 1995; Wood et al., 1996). The mutation in the HD gene involves an expansion of a CAG repeat at the 5′ end of the gene beyond its normal 10–35 repeat range (Albin and Tagle, 1995). The means by which this mutation causes preferential gene involvement is of unknown function, although several lines of evidence suggest that such chimeras should have been created. By contrast, Hdh−/− ES cells injected into blastocysts yielded offspring that were born and in adulthood were found to have Hdh−/− neurons throughout brain. The Hdh−/− cells were, however, 5–10 times more common in hypothalamus, midbrain, and hindbrain than in telencephalon and thalamus. Chimeric animals tended to be smaller than wild-type littermates, and chimeric mice rich in Hdh−/− cells tended to show motor abnormalities. Nonetheless, no brain malformations or pathologies were evident.

The apparent failure of aggregation chimeras possessing Hdh−/− cells to survive to birth is likely attributable to the previously demonstrated critical role of huntingtin in extraembryonic membranes. That Hdh−/− cells in chimeric mice created by blastocyst injection are under-represented in adult telencephalon and thalamus implies a role for huntingtin in the development of these regions, whereas the neurological dysfunction in brains enriched in Hdh−/− cells suggests a role for huntingtin in adult brain. Nonetheless, the lengthy survival of Hdh−/− cells in adult chimeric mice indicates that individual neurons in many brain regions do not require huntingtin to participate in normal brain development and to survive.

Key words: basal ganglia; cortex; development; Huntington’s disease; HD gene; colonization

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To determine whether neurons lacking huntingtin can participate in development and survive in postnatal brain, we used two approaches in an effort to create mice consisting of wildtype cells and cells without huntingtin. In one approach, chimeras were created by aggregating the 4–8 cell embryos from matings of Hdh−/− mice with wild-type 4–8 cell embryos. No chimeric offspring that possessed homozygous Hdh−/− cells were obtained thereby, although statistical considerations suggest that such chimeras should have been created. By contrast, Hdh−/− ES cells injected into blastocysts yielded offspring that were born and in adulthood were found to have Hdh−/− neurons throughout brain. The Hdh−/− cells were, however, 5–10 times more common in hypothalamus, midbrain, and hindbrain than in telencephalon and thalamus. Chimeric animals tended to be smaller than wild-type littermates, and chimeric mice rich in Hdh−/− cells tended to show motor abnormalities. Nonetheless, no brain malformations or pathologies were evident.

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the mating strategy described below. All studies were conducted in onic membranes (Le Dourain and McLaren, 1984). lines that were transgene. ES cell lines were derived from these blastocysts, and ROSA26 E3.5 blastocysts were obtained from this mating, 75% of which bore the ROSA26 in the abolition of expression for Duyao et al. (1995) mice involves a deletion of exons 4 and 5, resulting in telencephalon and thalamus. colonize hypothalamus, midbrain, and hindbrain and are scarce in telencephalon and thalamus. MATERIALS AND METHODS Subjects. Hdh+/− mice (Duyao et al., 1995) and ROSA26 mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred in our colony at the University of Tennessee. The Hdh disruption in the Duyao et al. (1995) mice involves a deletion of exons 4 and 5, resulting in the abolition of expression for Hdh. These mice were used together with the ROSA26 mice to create chimeras by the method of early embryo aggregation (with the embryos from both lines being at the 4–8 cell stage at the time of aggregation), as described below. Hdh−/− Hdh+/− chimeric mice also were created in the laboratory of Dr. Scott Zeitin by injecting embryonic stem (ES) cells into wild-type blastocysts (Dragatis et al., 1998). The Hdh nullizygous ES cells possessed a homozygous-targeted disruption of both Hdh alleles (Dragatis et al., 1998). The Hdh disruption in this case involved deletion of the promoter, exon 1, and flanking intronic sequences (HdhΔ1495), again resulting in the loss of Hdh expression (Zeitin et al., 1995). So that their detection could be facilitated in chimeric tissues, the Hdh−/− ES cells also bore a lacZ transgene that had been introduced into their genome by the mating strategy described below. All studies were conducted in accordance with National Institutes of Health and Society for Neuroscience policies on the ethical use of animals in research. Production of aggregation chimeras. Aggregation chimeras were created by standard methods (Goldowitz et al., 1992), with the goal of creating mice that consisted of a mixture of Hdh−/− cells and wild-type cells. Briefly, Hdh+/− females were superovulated and mated with Hdh−/− males. Plug-positive females were taken 2 d later, and their oviducts were flushed with medium to harvest the 4–8 cell stage embryos. The wild-type component of the chimera was derived from the 4–8 cell stage embryos of the ROSA26 strain of mice, which allows for identification of cells from the wild-type contribution to the chimeras by an X-gal histochemical procedure for the lacZ gene product (Friedman et al., 1991). Note that, in chimeras created by the method of aggregating two 4–8 cell stage embryos, the Hdh−/− cells would be able equally, in principle, to colonize all parts of the embryo, including the extraembryonic membranes (Le Douarin and McLaren, 1984). Production of chimeras by blastocyst injection. Hdh−/−/Hdh+/− ROSA26 ES cells were obtained by dissociating and culturing the inner cell mass from embryonic day 3.5 (E3.5) embryos that were the products of a mating strategy designed to produce embryos that possessed a homozygous Hdh deletion and also possessed the ROSA26 transgene. In the first step of this mating strategy Hdh−/− female mice were mated with males homozygous for the ROSA26 lacZ transgene. In the second step of the mating strategy the Hdh−/−/Hdh+/− ROSA26 oospairs were intercrossed, and E3.5 blastocysts were obtained from this mating, 75% of which bore the ROSA26 transgene. ES cell lines were derived from these blastocysts, and Southern and Western analyses were performed to identify those ES cell lines that were Hdh−/−, Hdh−/+ or ROSA26 ES cells were injected into wild-type C57BL/6 (B6) host E3.5 blastocysts via standard procedures, and the injected blastocysts were transferred into the uterine horns of pseudopregnant females. Any of the different lines of Hdh−/− ES cells described previously (A, B, or C) were used for these injections (Dragatis et al., 1998). These procedures have been described in detail previously (Dragatis et al., 1998). After birth, the chimeric mice were monitored and killed at various time points up to ~1 year of age. Note that, because ES cells preferentially colonize the embryonic ectoderm and poorly colonize extraembryonic tissues derived from the inner cell mass and completely fail to colonize extraembryonic tissues derived from trophectoderm, in the chimeras made by blastocyst injection the Hdh−/− cells are completely absent from trophectoderm derivatives and are mainly absent from the extraembryonic membrane derived from inner cell mass tissue (Beddington and Robertson, 1989). PCR genotyping of Hdh+/− mice used to generate embryos for aggregation chimeras. PCR genotyping was performed as described previously to identify Hdh+/− mice for breeding purposes (Duyao et al., 1995; Chen et al., 1997; Fusco et al., 1999). Genomic DNA extracted from tail biopsies was used to detect mice bearing an Hdh knock-out allele in their genome by PCR genotyping mice for Hdh and for the neomycin resistance selection cassette (neo) introduced during the creation of the Hdh deletion. The primers for the detection of huntingtin DNA in these PCR assays were 5′-CAAATGGTGCGTTGTCGTTG-3′ and 5′-GTCAGCT- GAGTGGACAGTTT-3′. The amplified Hdh DNA fragment has a size of 150 bp. The primers for the detection of neo DNA were 5′-CTTGGGTGGAAGGCTATTC-3′ and 5′-AGGTGAGATGCAGGAGAT-3′. The amplified neo PCR product has a size of 280 bp. One-half of a microliter of DNA template (250 ng/μl genomic DNA) was used, and the PCR for Hdh and neo for each animal was run simultaneously in the same glass-walled PCR tube. The PCR reaction solution contained the following: 2.0 μl of 10X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.6 μl of 25 mM MgCl2, 0.4 μl of 10 mM DNA template, 2.0 μl of 10 μM amounts of the four primers, 2.76 μl of DNA tracking dye, 4.66 μl of D H2O, and 0.08 μl of Taq polymerase. Contamination from extraneous DNA was checked by replacing the cellular template with water. Amplification was performed on a thermal cycler (Perkin Elmer, Waterrow, MA), typically using 25 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min for a total 30 cycles. After PCR amplification, aliquots of reaction product were analyzed by electrophoresis on ethidium bromide-impregnated 3% agarose gels. PCR genotyping of aggregation chimeras to detect Hdh−/− cells. So that cells that are homozygous for the Hdh knock-out could be detected, it was necessary to detect the presence of neo and the presence for the absence of Hdh. For distinguishing aggregation chimeras bearing homozygous Hdh knock-out cells from those bearing a hemizygous Hdh deletion, PCR genotyping by tail biopsy is inadequate, because both Hdh−/+ and Hdh−/− cells possess neo, and the possible presence of Hdh in wild-type cells in the tail biopsy would hide evidence of any Hdh−/− cells. Since cells potentially contributing to the formation of the chimera. Thus to detect the presence of Hdh−/− cells in the aggregation chimeras, we excised pure populations of ROSA26-negative cells (which come from the Hdh knock-out line) from the fixed livers of aggregation chimeras post-mortem. Liver slices (50 μm thick) from the aggregation chimeras that had shown a tail-snip PCR signal for neo were prepared and reacted with X-gal as described below. The liver slices were mounted on slides and examined with a dissecting microscope. Patches of cells that were devoid of X-gal labeling and were therefore from the Hdh knock-out strain were removed from the tissue with a glass micropipette. These cells were lysed with Proteinase K, and 1–2 μl of the digested material was assayed by PCR to determine whether it was negative for Hdh. Three primers were that allowed us to detect differentially the normal Hdh gene and the truncated form of the allele created by the targeted disruption of Hdh: (1) 5′-GTTGCAGTCCGATTCATCTC-3′; (2) 5′-AACGTAGCGGCCTT-3′; and (3) 5′-TGCCGGTCTCGGATCCGACGATC-3′. The first and second of these primers amplify a 350 bp fragment of wild-type Hdh containing intron 3 and exon 4, whereas the first and third primers amplify a 650 bp fragment of disrupted Hdh containing intron 3 and the neo insert. The PCR reaction solution contained the following: 2 μl of 10X PCR buffer, 1.2 μl of 25 mM MgCl2, 0.2 μl of 20 mM dNTPs, 1 μl of 20 μM amounts of the three primers, 2.76 μl of DNA tracking dye, 10.7 μl of D H2O, and 0.1 μl of 7.5 U Taq polymerase. Contamination from extraneous DNA was checked by replacing the cellular template with water. Amplification was performed on a thermal cycler (MJ Research), as described above, and aliquots of reaction product were analyzed by electrophoresis on ethidium bromide-impregnated 3% agarose gels. To ensure that chimeric mice genotyped as Hdh−/− by tail biopsy were in fact Hdh−/−, we performed genotyping of non-ROSA26 liver cells from these animals as well. Behavioral assessment of chimeras produced by blastocyst injection. Several simple motor/behavioral tests were performed on the chimeras produced by blastocyst injection of Hdh−/−/Hdh+/− ROSA26 ES cells (Dragatis et al., 1998). These included a limb-clasping assessment, a wire rod hanging test, an evaluation of the ability to cling to a wire cage being rotated at 240 rpm, and a cage activity test. These experiments have shown to be sensitive to the neurological/motor abnormalities seen in mutant mouse models of neurological disease (Mangiarini et al., 1996; Dragatis et al., 2000). Mice typically were tested once a month. For limb clasping, chimeric mice and nonchimeric littermates were suspended 30 cm above
an open cage and over a 1 min period were lowered toward the bottom of the cage. Mice that clasped their limbs within 5 sec of suspension and maintained the clasping during the entire descent were scored as showing a positive limb-clasping response. For the wire rod hanging test, mice were allowed to grasp a narrow wire rod (diameter, <0.25 cm) suspended 30 cm above a padded work surface and were observed for 1 min. Normal mice can maintain a grip with their forelimbs for the full test duration, so mice that fell within 30 sec of the onset of the test thus were scored as positive for neurological impairment. For the wire cage rotation test, mice were placed on top of a wire cage held ~30 cm above a padded work surface. Then the cage was rotated 180° over a period of 1 min and held for an additional 30 sec in the inverted position. Mice scoring positive for neurological impairment in this test were unable to climb to a position at the top of the rotating frame as it was turned and fell to the work surface (Crawley, 1999). For gait analysis, the hindpaws of mice were dipped in nontoxic fingernail paint and placed on a strip of paper between two guide walls. In the comparisons of chimeras and controls, the distance between successive paw prints and the width between each pair of prints were measured. Body weight was measured in the chimeric mice and a set of 10 age-matched C57BL/6 males and 10 age-matched C57BL/6 females at 1, 3, 6, and 9 months.

Tissue fixation. Under deep Avertin anesthesia, mice used for histochemical analysis were perfused transcardially with PBS (0.1 M sodium phosphate buffer, pH 7.4, with 0.9% NaCl), followed by 4% paraformaldehyde in 0.1 M PB. These mice included all chimeric mice created by the aggregation method, six ES blastocyst injection chimeras and their two B6 controls, and five nonchimeric ROSA26 mice used as controls to demonstrate the ability of the lacZ gene to be expressed ubiquitously throughout the nervous system in mice (Friedrich and Soriano, 1991). The brains and liver were removed and stored in a 20% sucrose/10% glycerol solution at 4°C. Livers from aggregation-chimera mice created by using ROSA26 as the wild-type strain were sectioned with a vibratome at 50 µm and used in genotyping, as noted above. The brains of the six ES blastocyst injection chimeras, their two B6 controls, and the control ROSA26 mice were sectioned frozen on a sliding microtome in the transverse plane at 35 µm. Each of these brains was collected as a separate pair of 0.1 M PB and 0.02% sodium azide and stored until processed for histology, immunohistochemistry. For four additional ES blastocyst injection chimeras the brains were removed and immersion fixed overnight in 0.2% paraformaldehyde in 0.1 M PIPES, pH 6.9, plus 2 mM MgCl2 and 5 mM EGTA, washed in PBS and cryo-preserved in 30% sucrose plus MgCl2, embedded in Tissu-Tek OCT compound (Fisher Scientific, Pittsburgh, PA), and frozen. These brains were sectioned in the sagittal plane with a cryostat and mounted on Superfrost Plus glass slides. A series of brain sections for each chimeric mouse created by the blastocyst injection method (and the B6 controls) was stained with cresyl violet or neutral red to study normal brain histology. Additional series were processed by using fluoro-jade labeling to detect any degenerating cells (Schmued et al., 1997). As positives were scored in the fluoro-jade studies, C57BL/6 mice were injected intraperitoneally with 1 mg of kainic acid per 30 gm of body weight. This dose was chosen because it gives stage 3 seizures and consistent hippocampal cell death (Schauwecker and Steward, 1997). These animals were perfusion fixed by using the same fixative as for the chimeric mice. Brains were cryosectioned, mounted on glass slides, and processed in parallel with the chimeric tissue for the fluoro-jade demonstration of degenerating neurons.

Demonstration of cell genotype via β-galactosidase histochemistry. One series of sections through the brain and/or liver of chimeric mice, the B6 control mice, and the nonchimeric ROSA26 control mice was processed for the β-galactosidase marker by using the procedure of Oberdick et al. (1994), in which sections are incubated at 30–35°C overnight in buffer (containing 5 mM of potassium ferriyanide and ferrocyanide, 2 mM magnesium chloride, 0.02% Nomidet P-40, and 0.01% sodium deoxycholate) with 0.1% X-gal substrate in dimethyl sulfoxide (Boehringer Mannheim, Indianapolis, IN). Slides were rinsed and counterstained with neutral red to identify and quantitatively labeled and unlabeled cells. Tissue was dehydrated and cleared in xylene; coverslips were applied with Permount. This approach identified the cells from the wild-type strain in aggregation chimeras in which the ROSA26 line was used as the wild-type strain and knock-out cells in the blastocyst injection chimeras (because Hdh−/− cells bore a lacZ transgene expressing β-galactosidase attributable to the mating strategy described above).

Immunohistochemistry. Immunohistochemical labeling was performed to confirm the absence of huntingtin in X-gal-labeled perikarya in the chimeras created by blastocyst injection and to characterize the effects of the homozygous Hdh deletion on these cells by assaying for perturbation in neuropeptide and neurotransmitter expression as well as for the expression of markers of regional or cellular stress. Conventional immunofluorescence or the peroxidase–anti-peroxidase (PAP) procedures were used (Anderson and Reiner, 1990, 1991; Figueredo-Cardenas et al., 1994). Tyramide signal amplification was used to enhance labeling for huntingtin (Fusco et al., 1999). In some cases the immunolabeling also was enhanced by prestaining the free-floating sections with a 30 min immersion in 10 mM sodium citrate buffer, pH 9.0, at 85°C (Jiao et al., 1999). To detect huntingtin, we used a mouse monoclonal antibody (Mab2170, Chemicon, Temecula, CA). The antibody was generated against amino acids1247–1646 of human huntingtin; its specificity for huntingtin in rodents and humans has been demonstrated previously (Bessert et al., 1995; Trotter et al., 1995; Fusco et al., 1999). Antigens that were screened to assess the normalcy of the blastocyst injection chimera brains possessing Hdh−/− cells included vasoactive intestinal polypeptide (VIP), tyrosine hydroxylase (TH), calbindin (CALB), parvalbumin (PARV), glial fibrillary acidic protein (GFAP), substance P (SP), methionine–enkephalin (ENK), neuropeptide Y (NPY), choline acetyltransferase (ChAT), and vasopressin (VP) (Loren et al., 1979; Kiyma et al., 1990; Reiner and Anderson, 1990; Armstrong et al., 1994; Figueredo-Cardenas et al., 1994, 1998; Kawaguchi et al., 1995; Karle et al., 1996; Elmquist et al., 1996; Figueredo-Cardenas et al., 1998). The specificity of the antisera that were used have been described in previous studies (Reiner, 1991; Armstrong et al., 1994; Figueredo-Cardenas et al., 1994, 1998; Karle et al., 1996).

RESULTS

Aggregation chimeras

From our efforts to create aggregation chimeras containing cells with a homozygous deletion of the huntingtin gene, 21 chimeric animals were born. Note that we would expect only 25% of our aggregation chimeras to possess Hdh−/− cells, because only 25% of the embryos from our mating of Hdh+/− males with Hdh+/+ females would be expected to be Hdh−/−.

To distinguish chimeras bearing Hdh−/− cells from those bearing Hdh+/− cells, we believed that PCR genotyping of DNA isolated from a tail biopsy would be inadequate, because wild-type cells in the tail biopsy would hide evidence of homozygous Hdh knock-out cells. Thus we used the strategy described in Materials and Methods whereby we excised pure populations of cells from the knock-out line from the fixed liver of postmortem chimeric mice. Of our 21 chimeric animals created by aggregation methods, 12 showed a positive signal for the neo cassette by PCR. The remaining mice (9) were chimeric assemblages of Hdh+/− cells from the knock-out strain and the wild-type strain. Of the 12 showing a positive signal for the neo cassette (indicating at least one Hdh null allele), none contained cells that were nullizygous for Hdh, and all neo-positive cells thus appeared to be Hdh+/−. The failure of aggregation chimeras with homozygous Hdh knock-out cells to be born is extremely unlikely to have occurred by chance, because of the 21 chimeric mice that were phenotyped, approximately five would have been expected to possess Hdh−/− cells (p < 0.05, by χ2 analysis). Based on these considerations, it seems that the chimeric mice possessing Hdh−/− cells presumably created by the aggregation method must not have come to term. It is of note that, of the 21 chimeric mice created by aggregation methods that were born, the percentage of contribution from the embryos derived from the Hdh+/− cross ranged from 5 to 95%. If the same percentage of chimerism can be assumed for the homozygous Hdh knock-out chimeras, who are presumed to have died in utero, it would suggest that even a very small percentage of Hdh−/− cells is incompatible with development to term.
**Blastocyst injection chimeras: Characteristics of Hdh<sup>+/−</sup> chimeras**

By contrast, blastocyst injection of Hdh<sup>+/−</sup> ES cells yielded viable chimeric mice (10 males, 13 females) that were able to live well into adulthood (Table 1). Chimeras were generated with any of three Hdh nullizygous ES cell lines (two female lines, clones I and B, and one male line, clone A). Southern blot data from organs of some killed chimeras suggested that the coat color chimers tended to reflect overall bodily chimism at the DNA level (the ES cell lines were derived from the 129 Sv/Ev background and gave rise to agouti animals, whereas the host blastocysts were obtained from C57BL/6 females with black fur). The chimeras were estimated to range from 20 to 75% in their contribution from the Hdh<sup>+/−</sup> ES-derived mouse strain by their coat color. The body weight of these chimeras ranged from 60 to 100% of wild-type mice, and body weight was significantly inversely correlated (r = 0.81), with the percentage of ES cell-derived contribution indicated by coat color (i.e., the more Hdh<sup>+/−</sup> cells, the lower the body weight of the chimera). The male chimeras never mated successfully; there were no pregnancies, and no vaginal plugs were detected in wild-type females housed with male chimeras. By contrast, female chimeras generated by blastocyst injection of Hdh<sup>+/−</sup> ES cells were able to mate successfully with wild-type males, because two transmitted the Hdh mutation to their progeny. Five male and two female chimeras showed a characteristic set of abnormal motor traits, notably clasping of the forelimbs and hindlimbs and curling of the body during the clasping, beginning at weaning (4 weeks of age). In addition, four of these chimeras, beginning at 4 weeks, could not grasp or support themselves well on an elevated wire rod, and, when placed on a wire cage that then was rotated slowly, the chimeras invariably fell off the cage (whereas control animals did not). By 20 weeks of age all seven of these chimeras failed the rod grasping and rotating cage grasping tests. A simple gait analysis that used inked hindpaws did not, however, reveal differences from controls. The abnormal animals also tended to be attacked and wounded by their littermates when housed with them, implying the symptomatic chimeric mice were less able to defend themselves.

Of the seven animals that exhibited the suite of severe behav-

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**Table 1. Characteristics of Hdh<sup>−/−</sup> ↔ wild-type chimeras created by blastocyst injection**

| Animal | Clone | Gender<sup>a</sup> | Percentage of Hdh<sup>−/−</sup> by coat color<sup>b</sup> | Age at death<sup>c</sup> | Percentage of wild-type weight at 6 months<sup>d</sup> | Motor abnormalities |
|--------|-------|--------------------|---------------------------|-------------------------|---------------------------------|---------------------|
| ES4    | A     | M                  | 20                        | 13 months              | 95                              | None                |
| ES19   | A     | F                  | 20                        | 13 months              | 103                             | None                |
| ES15   | I     | F                  | 25                        | 12 months              | 100                             | None                |
| ES5    | B     | F                  | 30                        | 12 months              | 102                             | None                |
| ES7    | B     | F                  | 30                        | 13 months              | 92                              | None                |
| ES8    | I     | F                  | 30                        | 13 months              | 90                              | None                |
| ES16   | A     | M                  | 30                        | 12 months              | 94                              | None                |
| ES18   | A     | F                  | 30                        | 11 months              | 95                              | None                |
| ES20   | A     | M                  | 30                        | 12 months              | 92                              | None                |
| ES12   | I     | M                  | 30                        | 8 months               | 70                              | Severe by 20 weeks<sup>e</sup> |
| ES14   | A     | M                  | 35                        | 11 months              | 93                              | None                |
| ES3    | I     | F                  | 40                        | 13 months              | 89                              | None                |
| ES17   | B     | F                  | 40                        | 12 months              | 91                              | None                |
| ES6    | A     | M                  | 40                        | 13 months              | 86                              | Slight clasping<sup>f</sup> |
| ES11   | A     | M                  | 45                        | 9 months               | 71                              | Severe by 20 weeks<sup>e</sup> |
| ES9    | I     | F                  | 50                        | 13 months              | 85                              | None                |
| ES13   | A     | M                  | 50                        | 9 months               | 70                              | Severe by 4 weeks<sup>e</sup> |
| ES1    | I     | F                  | 60                        | 13 months              | 82                              | Slight clasping<sup>f</sup> |
| ES10   | B     | F                  | 60                        | 6 months               | 65                              | Severe by 4 weeks<sup>e</sup> |
| ES23   | I     | M                  | 60                        | 9 months               | 73                              | Severe by 20 weeks<sup>e</sup> |
| ES2    | I     | F                  | 65                        | 13 months              | 80                              | Slight clasping<sup>f</sup> |
| ES22   | A     | M                  | 65                        | 8 months               | 63                              | Severe by 4 weeks<sup>e</sup> |
| ES21   | B     | F                  | 75                        | 7 months               | 60                              | Severe by 4 weeks<sup>e</sup> |

<sup>a</sup>Gender in the chimeras was influenced not only by the gender of the ES cell clone used but also by the gender of the host embryo and the degree of colonization by cells of either genotype of gender-determining body tissues.

<sup>b</sup>The mice are ordered by increasing ES cell line contribution to coat color to facilitate assessment of the association of ES composition with body weight and behavioral abnormality.

<sup>c</sup>Chimeric mice that displayed no symptoms were killed at ~1 year of age, whereas chimeric mice with behavioral symptoms were killed at the time they showed severe morbidity.

<sup>d</sup>A sample of 10 male and 10 female C57BL/6 mice was used as the age-matched and gender-matched wild-type control population. Adult body weight was stable in all animals before the onset of morbidity in those showing the severe abnormalities. Thus, 6 month body weight is shown for all animals, and none was showing morbidity at the time of measurement.

<sup>e</sup>Severe deficits on clasping and rod grasping tests at 4 weeks and severe deficit on cage rotation test at 20 weeks.

<sup>f</sup>Mild clasping was observed at 20 weeks, but no deficit was seen in the other tests at any time.

<sup>g</sup>Severe deficits on clasping, rod grasping, and cage rotation tests at 4 weeks.
ioral abnormalities noted above, six had a chimeric contribution ≥45% (based on coat color). By contrast, of the 16 chimeras that did not exhibit the severe behavioral abnormalities, 13 had a chimeric contribution <45% (based on coat color; Table 1). Among the three mice with a chimeric contribution ≥45% that did not show the suite of severe behavioral/motor abnormalities, two in fact showed mild clasping (one mouse with 60% coat color chimerism and one with 65%) but were unaffected in the other tests (Table 1). One further chimeric mouse (with 40% coat color chimerism) also had a mild clasping tendency but was unaffected in the other tests as well (Table 1). The data therefore suggest that the magnitude of the overall Hdh−/− contribution, as inferred from coat color, was associated with an increased likelihood of motor abnormalities, either severe or slight. To assess this apparent association, we assigned a score of 0 for no motor deficit; a 1 for a slight clasping defect; a 2 for showing clasping, poor rod grasping, and poor rotating cage grasping by 20 weeks; and a 3 for showing clasping, poor rod grasping, and poor rotating cage grasping by 4 weeks. With the use of this motor scoring scale, the percentage of ES cell contribution indicated by coat color is correlated significantly (r = 0.73) with the motor deficit. Nonetheless, it is evident that the severity of the behavioral abnormalities was not predicted inexorably by the percentage of coat color chimerism. For example, severe motor abnormalities were observed in one animal with only 30% coat color chimerism but were absent in three with ≥45% coat color chimerism. Such deviations from a strictly linear relationship between coat color chimerism and the magnitude of the motor abnormalities may have occurred because the extent of colonization by Hdh−/− cells of the key neural and/or the key extraneural regions that were needed to produce the observed suite of behavioral abnormalities was not reflected accurately in all cases by the abundance of Hdh−/− cells in the coat. Such an explanation is consistent with the evidence that the relative colonization of bodily tissues by the genotype different lineages comprising a chimeric animal can differ from tissue to tissue and animal to animal (Le Douarin and McLaren, 1984; Goldowitz et al., 1992; Kuan et al., 1997).

The seven mice showing the suite of behavioral abnormalities also exhibited severe weight loss and severe hypoactivity by 6–9 months of age and were killed at that time. The brains of four were immersion fixed overnight, as described in Materials and Methods, and stored frozen until processed as slide-mounted cryostat sections. Six of the chimeric mice that survived slightly beyond 1 year without showing the suite of severe behavioral abnormalities were perfused transcardially with fixative, as described in Materials and Methods, and the brains were processed histologically as free-floating slide-mounted sections.

**Blastocyst injection chimeras: Histological analysis of Hdh−/− mice**

The 10 chimeras and two B6 control mice were sectioned and analyzed histologically and histochemically. The brains of the chimeras appeared normal in gross morphology, cytoarchitecture, regional neuronal abundance, and ventricular outline (Fig. 1). The brains of the chimeras, however, typically were slightly smaller than those of the controls. In the 10 chimeras, numerous X-gal-labeled cells derived from the Hdh nullizygous ES cells injected at the blastocyst stage were found throughout the brain (Table 2; Fig. 1). No systematic differences were observed in the distribution of these Hdh−/− cells for the three injected lines of Hdh−/− embryonic stem cells (A, B, and I). Side-by-side comparisons of X-gal-labeled tissue and huntingtin-labeled tissue showed that regions rich in X-gal-labeled cells were poor in huntingtin-labeled cells, thereby confirming that X-gal-labeled cells were indeed Hdh−/−. This was particularly evident in the cornu ammonis of the hippocampus, in which alternating bands of X-gal-labeled and huntingtin-labeled neurons could be observed (Fig. 2). No X-gal-labeled cells were observed in the B6 control mice. Although X-gal-labeled Hdh−/− cells were found throughout the brain of all 10 chimeric mice, the Hdh−/− cells tended to be relatively scarce in the telencephalon (except for the hippocampus, in which they were consistently present), mainly absent from the thalamus and Purkinje cell layer of the cerebellum, and abundant in the epithalamus, preoptic region, hypothalamus, midbrain, granule cell layer of the cerebellum, and hindbrain (Table 2; Fig. 3). The abundance of these cells in these regions, however, varied from case to case. For example, in some cases Hdh−/− cells in hypothalamus and brainstem made up at least 50% of the cells, which was especially evident for the motoneuron pools in the case of the brainstem, whereas in others Hdh−/− cells made up no more than ~25% of the cells in the hypothalamus or brainstem (Table 2). In the latter cases Hdh−/− cells were nearly absent in the cerebral cortex, basal ganglia, and thalamus, whereas even in the former they typically did not include >10%
of telencephalic and thalamic cells. By contrast, in the nonchimeric ROSA26 control mice, X-gal labeling was observed in all cells throughout the brain, consistent with the previous observations of others (Friedrich and Soriano, 1991; Goldowitz et al., 2000).

Because the X-gal reaction product in the chimeric mice in many cases clearly could be localized to neurons (particularly in the case of large neurons for which the shape was unmistakable because the X-gal reaction product followed the cellular outline; Fig. 3), our results show that Hdh−/− neurons can survive in the chimeric brain for >1 year and that, up to an abundance of 50% Hdh−/− cells in midbrain and hindbrain, brain development seems normal. Three of the four chimeras showing the behavioral abnormalities and morbidity that were examined histologically, however, were enriched in hypothalamic and brainstem Hdh−/− cells (estimated to be 50–75% for the brainstem motoneuron pools, for example, in these mice). By contrast, none of the chimeric mice who showed no morbidity up to 1 year after birth and who were examined histologically possessed >50% Hdh knockout cells in both hypothalamus and motoneuron pools (Table 2). This suggests that >50% colonization of hypothalamus and brainstem by Hdh−/− cells may have played a role in the behavioral abnormalities and morbidity observed in at least some chimeric animals.

We used various immunomarkers to assess the normalcy of brain functional architecture. In light of the prominent involvement of the basal ganglia in HD, the possible effect of Hdh−/− cells on the striatum and its projection systems was of particular interest. Although the chimeric animals varied in their abundance of Hdh−/− cells in the striatum (from negligible to ~15%), we observed no evident differences between chimeric mice and wild-type mice in the distribution or abundance of calbindin-containing projection neurons or parvalbumin-containing interneurons in the striatum (Figs. 4, 5), both of which are affected in HD (Kiyama et al., 1990; Harrington and Kowall, 1991). Additionally, the abundance of enkephalinergic striatal fibers in the globus pallidus and of substance P-containing fibers in the entopeduncular nucleus and substantia nigra appeared no different from those in the B6 control mice (see Fig. 4), suggesting further that striatal projection neurons, which are affected prominently in HD (Reiner et al., 1988; Albin et al., 1990a,b, 1992; Richfield et al., 1995; Sapp et al., 1995), were unaffected in the chimeric mice. Similarly, a high abundance of Hdh−/− cells in the hypothalamus was not associated with any evident abnormality in the hypothalamic distributions of vasopressin, VIP, tyrosine hydroxylase, or NPY, and a prevalence of Hdh−/− neurons in brainstem motoneurons was not associated with any evident loss of choline acetyltransferase labeling of these neurons or any alteration in the abundance of these neurons (Figs. 6, 7). Consistent with the absence of any brain abnormalities, as assayed by neupeptide or neurotransmitter-related enzyme content, neither immunolabeling for GFAP (see Figs. 5, 7) nor staining with fluoro-jade, a marker of neurodegenerative changes, revealed any signs of pathology in the chimeric brains. It is noteworthy that this was true for both the chimeras showing morbidity by 6–9 months as well as those surviving with no signs of ill health until the time of death at ~1 year. By contrast, the hippocampus of the kainate-injected C57BL/6 mice showed large numbers of degenerating fluoro-jade-positive pyramidal neurons.

DISCUSSION

To understand better the significance of huntingtin in neuronal function, we overcame the embryonic lethality of homozygous knock-out of the HD gene homolog in mice by a chimeric strategy. Our findings reveal several important points regarding the role of huntingtin in neural development and neuronal survival, as discussed below.

Comparison to previous findings by others

Our failure to produce viable aggregation chimeras possessing cells with homozygous Hdh deletion may stem from the previously demonstrated role of huntingtin in the extraembryonic membranes, particularly those derived from trophectoderm, during early development (Dragatiss et al., 1998). The apparent participation of huntingtin in vesicular trafficking (DiFiglia et al., 1995; Sharp et al., 1995; Wood et al., 1996) may be important in

Table 2. Blastocyst injection chimeras: Distribution of Hdh−/− cells in brain and traits of chimeric mice examined histologically

| Animal  | ES clone | ES1 | ES2 | ES3 | ES4 | ES5 | ES6 | ES7 | ES8 | ES9 | ES10 | ES11 | ES12 | ES13 |
|---------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|
| ES clone |          |     |     |     |     |     |     |     |     |     |      |      |      |      |
| Percentage of KO by coat | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% | 40% |
| Age at death | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months | 13 months |
| Behavioral abnormalities | Slight clasping | Slight clasping | None | None | None | None | None | None | None | None | None | None | None | None |
| KO cell abundance |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Neocortex | – | + | + | – | – | + | – | – | – | + | – | – | – | + |
| Hippocampus | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Striatum | – | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Globus pallidus | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Thalamus | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Hypothalamus | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Midbrain | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Cerebellar GCL | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Cerebellar PCL | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Brainstem motoneurons | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Hindbrain | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

–, <1% KO cells; +, 1–10% KO cells; ++, 11–25% KO cells; ++++, 26–50% KO cells; ++++, >50% KO cells; ND, Not done.

KO, Hdh−/−; GCL, granule cell layer; PCL, Purkinje cell layer.
the transport of nutrients across the extraembryonic membranes, and disruption of this function may be the basis of the embryonic lethality of homoygous huntingtin deletion (Dragatsis et al., 1998). Huntingtin with a polyglutamine expansion in the range causing HD does not disrupt this function critically, because humans with a homozygous HD mutation do not exhibit embryonic lethality (Wexler et al., 1987; Myers et al., 1989; Gusella and MacDonald, 1996) and because a transgene expressing huntingtin with such a polyglutamine expansion rescues Hdh null mice from embryonic lethality (Hodgson et al., 1999; Leavitt et al., 2000). Additionally, one normal huntingtin allele is sufficient in humans or mice for normal development and postpartum life (Ambrose et al., 1994; Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995; Persichetti et al., 1996). By contrast, our inability to produce viable chimeras containing Hdh /−− cells by using the method of early embryo aggregation suggests that even limited colonization of the extraembryonic membranes by Hdh /−− cells may impair their function so as to be lethal to the developing embryo. Note, however, that we did not investigate the stage beyond which our aggregation chimeras with Hdh /−− cells failed to develop. Deficiencies in huntingtin also are known to promote defects in proliferation (White et al., 1997), and it may be that such a defect placed the 4−8 Hdh /−− cells from the Hdh knock-out strain at a disadvantage relative to the 4−8 Hdh +/+ cells from the wild-type strain in their ability to proliferate and populate chimeras after aggregation.

The results of our studies on chimeras created by the blastocyst injection method, in which the lethal effects of Hdh /−− cells in the extraembryonic membranes are avoided (Dragatsis et al., 1998), demonstrate that Hdh /−− cells can participate in the normal formation of the brain and can develop into neurons with normal morphology and transmitter content that survive for >1 year with no evident signs of brain pathology. These results extend on recent data showing that Hdh /−− ES cells transformed into a neuronal phenotype in vitro can survive for several weeks and show typical neuronal electrophysiological traits (Metzler et al., 1999). We did, however, find evidence that Hdh /−− cells do not colonize equally and/or thrive in all brain areas. In particular, we observed that neurons and/or glia derived from the Hdh /−− ES cells tended to colonize preferentially the hypothalamus, midbrain, granule cell layer of cerebellum, and hindbrain, with only a sparse occupancy of cerebral cortex, basal ganglia, thalamus, and Purkinje cell layer of cerebellum. Our studies of lacZ expression in control ROX/A26 mice indicate that this paucity of X-gal-labeled cells in some brain regions in the blastocyst injection chimeras is not a false negative stemming from poor lacZ expression by ES cells that had colonized these regions. Thus our findings raise either of two possibilities. First, it may be that some peculiarity of our ES cells independent of their Hdh genotype resulted in this differential colonization. Although there is evidence that ES cells can show a tendency to colonize CNS differ-
entially in chimeric animals (Kuan et al., 1997), the pattern has been found to vary randomly from one chimeric animal to the next even for the same ES cell line. By contrast, we observed the same consistent pattern of preferential hypothalamic, midbrain, and hindbrain colonization by ES cells for each of our three independently derived lines of \textit{Hdh}^{-/-} ES cells, and the consistency of preferential colonization was greater than reported to occur for a single ES cell type (Kuan et al., 1997). Thus the second, and more likely, interpretation of the consistent paucity of \textit{Hdh}^{-/-} ES cell progeny in cerebral cortex, basal ganglia, thalamus, and the Purkinje cell layer of cerebellum is that huntingtin may be needed for cells to migrate to, proliferate in, and/or survive extensively within these regions.

Thus individual cells in many brain regions may not need huntingtin to differentiate into neurons that survive and function normally, whereas in other brain regions many cells may need to express normal levels of huntingtin if development is to proceed normally for that region. For example, brain development may

\begin{itemize}
  \item Figure 4. High-magnification images of transverse sections through dorsomedial striatum showing the distribution and abundance of \textit{Hdh}^{-/-} cells (as visualized by X-gal labeling, followed by a light neutral red counterstain) in the striatum of a chimeric mouse created by blastocyst injection of \textit{Hdh}^{-/-} ES cells. Shown is the striatum of a chimeric mouse, who displayed no ill health up to 1 year of age (A), compared with the striatum of a wild-type mouse in which no \textit{Hdh}^{-/-} cells are present (B). C, D. The presence of \textit{Hdh}^{-/-} cells in the striatum of the chimeric mouse shown in A has not produced any evident abnormality in the enkephalinergic striatal output fibers (ENK; visualized by DAB immunolabeling) within the ipsilateral globus pallidus (GP) of the chimeric mouse (C). Medial is to the left and dorsal to the top in all images.

  \item Figure 5. High-magnification images of transverse sections through dorsomedial striatum of the same chimeric and wild-type animals as shown in Figure 4. The presence of \textit{Hdh}^{-/-} cells in the striatum of the chimeric mouse has not produced any evident abnormality in the labeling of calbindering striatal perikarya (A, B) or any evidence of neuropathology in the striatum, as shown by the absence of any upregulation of glial fibrillary acid protein (GFAP) in the striatum (C, D). Medial is to the left and dorsal to the top in all images.

  \item Figure 6. High-magnification images of transverse sections through the paraventricular nucleus of the hypothalamus showing the distribution and abundance of \textit{Hdh}^{-/-} cells in striatum of a chimeric mouse created by blastocyst injection of \textit{Hdh}^{-/-} ES cells (ES8). Shown is the striatum of a chimeric mouse, who displayed no ill health up to 1 year of age (A), compared with the paraventricular nucleus of a wild-type mouse in which no \textit{Hdh}^{-/-} cells are present (B). The \textit{Hdh}^{-/-} cells in A are visualized by X-gal labeling, and neuronal cytoarchitecture in both A and B is visualized by neutral red counterstaining. C, D. The presence of \textit{Hdh}^{-/-} cells in the paraventricular nucleus of the chimeric mouse shown in A has not produced any evident abnormality in the vasopressinergic (VP) neurons (visualized by DAB immunolabeling) of the ipsilateral paraventricular nucleus of the chimeric mouse. Medial is to the left and dorsal to the top in all images.
\end{itemize}
have been seemingly normal even in the hypothalamus and brainstem of chimeras in which $\geq 50\%$ of the resident neurons were Hdh$^{-/-}$, because huntingtin plays no major role in the development and/or functioning of these regions. By contrast, huntingtin clearly seems to be critical for cortical and striatal development, because mouse mutants in which huntingtin is expressed at one-third of wild-type levels exhibit defective neurogenesis, profound malformations of cortex and striatum, ventricular enlargement, and agenesis of fiber tracts (White et al., 1997). Consistent with a regionally differential role of huntingtin in neural development, cortex and striatum express higher levels of huntingtin than do hypothalamus and brainstem (Li et al., 1993; Landwehrmeyer et al., 1995; Sharp et al., 1995; Bhide et al., 1996; Sapp et al., 1997; Fusco et al., 1999). The absence of forebrain developmental abnormalities in our chimeras with Hdh$^{-/-}$ cells may be a consequence of the relatively low colonization of cortex and striatum by Hdh$^{-/-}$ cells. This low colonization could stem from impaired proliferation of Hdh$^{-/-}$ cells during development or from the death of these cells. Note, however, that we did not see signs that cell loss had occurred recently in cortex, basal ganglia, thalamus, or Purkinje cell layer in the chimeras. Thus if the paucity of Hdh$^{-/-}$ neurons in telencephalon, thalamus, and Purkinje cell layer reflects their failure to survive in these regions, this loss had to occur at an early enough point in development for the Hdh$^{-/-}$ neuroblasts to have been replaced by wild-type neuroblasts. It is also possible that the reduced brain size seemingly typical of the chimeras masked evidence of early neuron loss.

The chimeric mice created by the blastocyst injection method showed a reduced body size proportional to the bodily abundance of Hdh$^{-/-}$ cells implied by coat color, and the chimeras most enriched in Hdh$^{-/-}$ cells additionally tended to show motor abnormalities and morbidity before 1 year of age. The basis of these abnormalities is uncertain, but the findings do suggest that an excess of Hdh$^{-/-}$ in some key neural (or extraneural) regions compromises their function in some currently unknown way. For example, the reduced body size characteristic of the blastocyst injection chimeras could stem from abnormalities in feeding and growth regulation because of colonization by Hdh$^{-/-}$ cells of neural and extraneural regions critical to these processes. The mice with the behavioral abnormalities and premature morbidity tended to be those in which Hdh$^{-/-}$ cells in general, as inferred from coat color, made up $\geq 45\%$ of the cells in the body. Given, however, that colonization of any individual tissue by Hdh$^{-/-}$ cells may have deviated from the degree of colonization suggested by coat color (Le Dourain and McLaren, 1984; Goldowitz et al., 1992), the bodily tissues critical to the occurrence of the abnormalities may not have been colonized highly in all chimeras that were $\geq 45\%$ Hdh$^{-/-}$ by coat color, or they may have, in fact, been colonized highly in chimeras with $< 45\%$ Hdh$^{-/-}$ by coat color. This may explain the absence of abnormalities in some chimeras $\geq 45\%$ Hdh$^{-/-}$ by coat color and their presence in one with $< 45\%$ Hdh$^{-/-}$ by coat color. The identity of the neural or extraneural regions for which the high colonization by Hdh$^{-/-}$ cells is critical to the occurrence of the behavioral/motor abnormalities in the blastocyst injection chimeras is uncertain. In three of the four mice with the behavioral abnormalities that were examined histologically, $> 50\%$ of the neurons in hypothalamus and brainstem motoneuron pools were found to be Hdh$^{-/-}$, suggesting their involvement in the abnormalities in at least some chimeric mice. The precise basis of the behavioral/motor abnormalities, however, will require focused study of the relationship between the percentage of colonization of various bodily tissues and the abnormalities in a larger number of animals than was possible in the present study.

Finally, the infertility of the male blastocyst injection chimeras may be behavioral, or it also could be attributable to problems in the testes. In this light, it is interesting that mice with a conditional deletion of Hdh under the control of a calmodulin-dependent kinase II (CaMKII) promoter (CaMKII is expressed late in development and thereby allows mice to evade the embryonic lethality of homozygous Hdh knock-out) are infertile because of a very low sperm count (Dragatsis et al., 2000). Similarly, mice possessing transgenic insertions of CAG-expanded forms of the human HD gene against a nullizygous Hdh background have been shown to undergo spermatocyte degeneration (Leavitt et al., 2000). By contrast, Hdh$^{-/-}$ males mated with wild-type females can pass on the Hdh mutation to their offspring, implying that sperm (which are haploid) lacking huntingtin can survive and fertilize eggs (Duyao et al., 1995; Nasir et al., 1995; Zettl et al., 1995). Thus huntingtin may not be needed for the viability of sperm, but it may play some critical role in the

Figure 7. High-magnification images of transverse sections throughpons showing the distribution and abundance of Hdh$^{-/-}$ cells in the facial nucleus in a chimeric mouse created by blastocyst injection of Hdh$^{-/-}$ ES cells. Shown is the facial nucleus in a chimeric mouse, who displayed no ill health up to 1 year of age (A), compared with facial nucleus of a wild-type mouse in which no Hdh$^{-/-}$ cells are present (B). The Hdh$^{-/-}$ cells in A are visualized by X-gal labeling, and neuronal cytoarchitecture in both A and B is visualized by neutral red counterstaining. C, D. The presence of Hdh$^{-/-}$ cells in the facial nucleus of the chimeric mouse shown in A has not produced any evident abnormality in the facial motoneurons (visualized by DAB immunolabeling for choline acetyltransferase) within the ipsilateral facial nucleus of the chimeric mouse. High-magnification images of transverse sections through facial nucleus of the same chimeric and wild-type animals as shown in A and B reveal that the presence of Hdh$^{-/-}$ cells in the facial nucleus of the chimeric mouse has not produced any upregulation of GFAP in the facial nucleus (E, F). Medial is to the left and dorsal to the top in all images.
viability of spermatogonia or primary spermatocytes or in spermatogenesis itself.

Implications for the pathogenesis of HD

A gain of function associated with the HD mutation is the aggregation of the N-terminal fragment of mutated huntingtin within neuronal nuclei and cytoplasm (DiFiglia et al., 1997; Li and Li, 1998; Martindale et al., 1998; Gutekunst et al., 1999; Maat-Schieman et al., 1999). Although considerable attention has focused on the possibility that these aggregates are a key pathogenic event in HD (Davies et al., 1997; DiFiglia et al., 1997; Kim and Tanzi, 1998; Saudou et al., 1998; Sidossia, 1998), the means by which they might lead to neuronal death remains uncertain (Cha et al., 1998; Hackham et al., 1998; Sidossia, 1998). The possibility that the aggregates may act, at least in part, by inactivating both mutant and normal huntingtin has been raised by recent evidence that the aggregates that form in HD and in transgenic animal models of HD can sequester normal-length polyglutamine-containing proteins, including huntingtin (Cha et al., 1999; Narain et al., 1999; Preisinger et al., 1999; Wheeler et al., 1999; Cattaneo et al., 2001). This has led to the recent suggestion that the HD mechanism of action might receive a contribution from a late-onset inactivation of normal huntingtin (Cattaneo et al., 2001). Such a possibility is consistent with the finding that huntingtin appears to exert an anti-apoptotic effect in cultured striatal neurons subjected to serum deprivation or metabolic stress (Rigamonti et al., 2000). Our finding that neurons that are Hdh<sup>−/−</sup> appear to be defective in their ability to colonize cortex, striatum, thalamus, and Purkinje cell layer of cerebellum suggests that neurons in these regions may require huntingtin more critically to develop and/or survive normally. If, in fact, these neuron types require huntingtin for long-term survival in adult brain, the fact that they are all affected in HD (Roos, 1986) would be consistent with a contribution of late-onset inactivation of normal huntingtin to HD pathogenesis. Nonetheless, this possibility would leave unexplained why striatal neurons are so much more vulnerable and cortical neurons somewhat more vulnerable than thalamic neurons and Purkinje cells in HD (Roos, 1986). It may be that striatal and, less so, cortical neurons more greatly require huntingtin for survival than do thalamic neurons and Purkinje cells. Such a possibility is consistent with the preferential morbidity of Hdh<sup>−/−</sup> neurons in cortex and striatum in postweaning mice in which the Hdh knock-out is expressed beginning late in embryonic development (Dragatsis et al., 2000). On the other hand, it is also possible that inactivation of normal huntingtin plays little, if any, role in HD pathogenesis.

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