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Huntington's disease alters human neurodevelopment

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Although Huntington's disease is a late-manifesting neurodegenerative disorder, both mouse studies and neuroimaging studies of presymptomatic mutation carriers suggest that Huntington's disease might affect neurodevelopment. To determine whether this is actually the case, we examined tissue from human fetuses (13 weeks gestation) that carry the Huntington's disease mutation. These tissues showed clear abnormalities in the developing cortex, including mislocalization of mutant huntingtin and junctional complex proteins, defects in neuroprogenitor cell polarity and differentiation, abnormal ciliogenesis, and changes in mitosis and cell cycle progression. We observed the same phenomena in Huntington's disease mouse embryos, where we linked these abnormalities to defects in interkinetic nuclear migration of progenitor cells. Huntington's disease thus has a neurodevelopmental component and is not solely a degenerative disease.

Huntington's Disease (HD) is a neurodegenerative disease that is part of the larger family of "proteopathies," which includes the polyglutamine diseases, amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease. These diverse disorders share a delayed onset in mid-adulthood or later despite the expression, at least in hereditary cases, of the disease-driving protein from the first days of life. This raises the question of whether early events might set the stage for later disease. For example, huntingtin (HTT), the protein mutated in HD, is essential for development (1–3). The mutant HTT (mHTT) impairs neural progenitor cell division, neuronal migration and maturation (4–6), giving HD mice a thinner cortex (7). The fact that expression of either mHTT or hypomorphic HTT solely during early life is sufficient to produce HD features in adult mice strongly suggests that there is a developmental component to the disease (8, 9).

In support of this notion, human neuroimaging studies have revealed smaller intracranial volume in HD mutation carriers as young as seven years of age (10, 11). Loss of cortical volume takes place long before any symptoms appear, and defects in the corticostriatal network lead to striatal dysfunction and degeneration (12–15). Studies in neurons derived from HD human induced pluripotent stem cells (iPSC) have identified changes in gene expression that sup-

port an altered developmental program (16, 17), and mHTT alters neuronal identity in cortical populations of HD brain organoids (18). But does mHTT affect early human development? And if so, how early? To answer these questions, we recruited HD mutation carriers who sought prenatal testing in order to determine whether the fetus carried an HD-causing mutation.

Mutant huntingtin mislocalizes in human and mouse embryos

We were able to procure rare intact cortical tissues from four HD mutation carrier fetuses and four healthy controls at age gestation week 13 (GW13) (table S1). At this developmental stage, the cortical neurons that project to the striatum and later deteriorate in HD are arising from the division of progenitor cells at the ventricular zone. These apical progenitors extend processes toward both the apical and basal surfaces of the neuroepithelial wall, and their nuclei move back and forth between surfaces in concert with cell-cycle progression in a process known as interkinetic nuclear migration. This process, common to all developing pseudostratified neuroepithelia (19, 20), maintains the balance between progenitor renewal and differentiation by controlling when and how much apical progenitor nuclei

are exposed to proliferative versus neurogenic signals.

We first examined the expression pattern of HTT at the ventricular zone of the GW13 cortex using an antibody that recognizes both HTT and mHTT (4C8; Fig. 1, A and B, and fig. S1A). In wild type tissues, HTT staining demarcated the apical surface of the ventricular zone and spread diffusely throughout the basal region. In cortical tissue of HD mutation carriers, however, HTT staining concentrated at the apical endfeet (the apical surface of the processes).

Given the preciousness of the human tissue, we turned to mice to further investigate these observations, using embryonic day 13.5 (E13.5) mouse embryos, which correspond to GW13 in human neurodevelopment. We studied an HD knock-in mouse model in which the first exon of the *HTT* gene is replaced by human exon 1 carrying 111 CAG repeats (Hdh^{Q111/Q111}) (21). Immunostaining coronal sections revealed a pattern of HTT expression that paralleled our observations in human fetuses (fig. S1B). To determine the distribution of mHTT specifically, we used another HD knock-in mouse model in which Flag tags are inserted in the N-terminal of wild type HTT (Hdh^{F7Q/+}) or mutant HTT carrying 140 CAG repeats (Hdh^{F140Q/+}) (22) (fig. S1C). The Flag labeling showed that mHTT localized to the apical surface and was decreased in the basal region.

Mutant huntingtin impairs endosome secretion and recycling

Apical progenitors maintain their polarity through endocytosis and trafficking from the trans-Golgi network to the plasma membrane at the apical endfeet (19). In HD, both endocytosis and Golgi-membrane trafficking are dysregulated (23). Because one of HTT's main functions is to transport vesicles, we used markers of the endosomal pathway to map the subcellular localization of HTT in HD and gauge whether transport is affected this early in HD.

We stained for Calnexin (a marker of the endoplasmic reticulum), GRASP65 (Golgi reassembly-stacking protein of 65 kDa, to mark the cis-Golgi network), TGN38 (trans-Golgi network integral membrane protein 38), EEA1 (early endosome antigen 1) and transferrin receptor (recycling endosomes). In control samples, HTT co-localized partially with these markers (figs. S2 and S3). In both human and mouse HD samples, however, HTT strongly co-localized with TGN38, EEA1 and transferrin receptor, and to a lesser extent with Calnexin and GRASP65. These results suggest that mHTT hinders endosomal trafficking in apical progenitors, even at this very early stage of development.

Mutant huntingtin disrupts neuroepithelial junctional complexes

Apical endfeet contain junctional complexes (19, 24) composed of tight- and adherens-junction proteins, including

ZO1, PAR3, NCAD and β -catenin (25), that link neighboring progenitors to each other, thereby sealing the neuroepithelium. Because HTT regulates the trafficking of these proteins, which are dysregulated in HD (6, 26–30), we hypothesized that mHTT hinders the correct positioning of these junctions, which would diminish the integrity of the neuroepithelium. As predicted, HTT partially co-distributed with ZO1, PAR3, β -catenin and NCAD at the apical endfeet of human GW13 control and mouse E13.5 neuroepithelium (figs. S4 and S5A). The levels of ZO1, NCAD and β -catenin were high at the apical surface of the human control ventricular zone and even higher in the HD human and mouse, with a concomitant reduction in these proteins in the basal region (Fig. 1, C to F, and fig. S5, B to E). PAR3 was also misregulated in HD but in a different pattern from these other proteins: its levels were down-regulated, so its demarcation of the apical surface in control samples was diminished, rather than intensified, in HD.

To better understand how junctional complexes in individual apical endfeet are affected in HD, we electroporated E13.5 control and HD mouse embryos *in utero* with a pCAG-GFP construct and performed immunohistochemistry on E15.5 coronal sections (Fig. 2, A and B). At this stage in mouse and at the corresponding stage GW16 in human (table S1), the mislocalization of HTT and junction proteins in HD apical progenitors persisted (figs. S6 and S7). Indeed, GFP-expressing knock-in HD progenitors, but not controls, showed a bright line of HTT along the apical surface (Fig. 2B). In control embryos, ZO1, NCAD and β -catenin immunostaining marked the sides of the apical endfeet; PAR3 staining was more apical (Fig. 2C). In Hdh^{Q111/Q111} embryos, ZO1, NCAD and β -catenin spread throughout the apical endfoot and PAR3 staining was diminished. These observations were corroborated by immunoblotting protein extracts from Hdh^{Q7/Q7} and Hdh^{Q111/Q111} E15.5 cortices (Fig. 2D). The levels of NCAD and β -catenin were similar in control and HD conditions, but ZO1 and PAR3 protein levels were lower in the mutant mice. Co-immunoprecipitation showed that HTT associates with ZO1, PAR3 and β -catenin, but these interactions were disrupted in HD (Fig. 2E and fig. S8).

Mutant HTT alters progression through the cell cycle

The integrity of the apical junctional complexes is essential for progression through interkinetic nuclear migration, when the nuclei of progenitor cells born at the apical surface move toward the basal side during the G1 phase of the cell cycle, enter and complete the S-phase, then return to the apical surface, where they undergo division (19, 20, 31, 32). Given that the junctional complexes do not form properly with mHTT, we examined cell cycle progression in the apical progenitors.

To measure apical (G1 phase) and basal (G2 phase)

movements *in vivo*, we used the fluorescent ubiquitination-based cell cycle indicator (FUCCI), which tracks the expression of markers of the different phases of the cell cycle (33). We electroporated wild type E13.5 embryos with plasmids encoding the chromatin licensing and DNA replication factor 1 (CDT1)-mKO2 and Geminin-GFP, then carried out time-lapse imaging on acute cortical slices two days after *in utero* electroporation (Fig. 3A) so that we could distinguish cycling progenitors in G1 from neurons exiting the cell cycle and migrating away from the ventricular zone (fig. S9A and movie S1). As expected, CDT1 levels peaked during G1 and fell upon entry into S phase, whereas geminin levels were high during S phase and G2 (fig. S9B and movie S2). The velocities of nuclear movement in G1 and G2 in control cells were as previously reported (34) (Fig. 3, B, C, and E, and movies S3 to S6), but in *Hdh*^{Q111/Q111} embryos, migrating nuclei moved more slowly in both G1 and G2, causing these phases to lengthen while the G1/S phase transition was shortened (Fig. 3, D and E, and movies S7 and S8). We next immunostained cortical sections of *Hdh*^{Q111/Q111} embryos and GW13 HD carrier fetuses with antibody against phosphohistone 3 (PH3), a marker of mitosis, and evaluated the mitotic index (Fig. 3F and Fig. 4, A and B). HD mice and human mutation carriers had roughly half the mitotic index of controls. In HD, therefore, the pool of proliferating cells is diminished.

Mutant HTT biases neurogenesis toward the neuronal lineage

The cell cycle correlates with the assembly (during G0) and disassembly (at the onset of M-phase) of the primary cilium at the apical progenitor endfeet (19, 32). Immunostaining human and mouse control and HD samples with the cilia marker Arl13b, a member of the ADP-ribosylation factor-like family, revealed that both the length and density of the cilia were greater at the apical area of the developing cortex in HD mouse and human samples compared to controls (Fig. 4C and fig. S10A), which confirms that the cells are not progressing through the cell cycle properly (20). Since longer G1 phase and shorter G1/S transition characterize progenitors committed toward the neuronal lineage (32, 35), we asked whether mHTT favors the production of apical over basal progenitors.

We evaluated cilia orientation by labeling brain sections with F-actin (to delineate the apical surface) and Arl13b and γ -tubulin (to label the basal body) (Fig. 4D and fig. S10B). The proportion of baso-lateral cilia, which signal the generation of basal progenitors (36), was greater in HD than in controls. To discriminate between apical progenitors and basal progenitors, which are more engaged in the neuronal lineage (37), we labeled for the transcription factors PAX6 and TBR2, respectively. HD mouse and human samples

showed a greater proportion of basal progenitors at the ventricular zone, subventricular zone, and inner subventricular zone than controls (Fig. 4E and fig. S10C).

Discussion

Our data show that mHTT mislocalizes at junctional complexes, disrupts the polarity of human and mouse neuroepithelium, and interferes with the cell cycle of apical progenitors, leading to fewer proliferating cells and more neural progenitors prematurely entering lineage specification. This is consistent with previous evidence that HTT regulates cellular adhesion, polarity, and epithelial organization (27). In the presence of mHTT, the epithelial-mesenchymal transition is accelerated (28). It is possible that mHTT contributes to cellular disorganization through other means as well, such as by interfering with the orientation of the mitotic spindle (7). Given that HTT also establishes apical polarity in the mammary epithelium, where it forms a complex with PAR3, aPKC, and RAB11A and ensures the apical translocation of PAR3-aPKC through RAB11A (29), we speculate that HTT may act to maintain epithelial cell polarity throughout the body.

A recent neuroimaging study found that the posterior Sylvian fissure, normally asymmetrical between the right and left hemispheres, lacks asymmetry in the HD population studied (38). Since the Sylvian fissure appears early *in utero*, the authors concluded that this abnormal symmetry arises during fetal development. Our results show that mHTT does alter very early stages of brain development in human HD, despite the fact that the samples we analyzed were from mutation carriers with small pathological expansions (39, 40 and 42 repeats) that would typically cause later manifestations of HD. The defects we observed likely render the cortico-striatal circuitry more vulnerable to the later dysfunctions characteristic of HD (23), as proposed for another polyglutamine disease, spinocerebellar ataxia type 1 (39). The path to degeneration is complex, however, and weaves together both pathogenic and compensatory mechanisms. For example, a recent study showed that HD mutation carriers around the age of six years show compensatory hyperconnectivity between the striatum and cerebellum; this initially enlarges the striatum but the metabolic load soon overwhelms it, the connections are rapidly lost, and the striatum atrophies well before the onset of motor symptoms (40).

It is beyond doubt now that neurodegenerative diseases can have a developmental component. For HD, this discovery opens the door for future studies to identify molecular treatments. For example, the HD iPSC Consortium characterized isoxazole-9 after finding it reverts abnormal neuronal differentiation in HD-derived pluripotent stem cells (17). It may be that treatment should be given very early in

life; it remains to be seen whether reducing mHTT levels in adulthood, even in the prodromal stage, would be sufficient to forestall symptom progression, since the brain circuitry is already altered.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S10

Table S1

Movies S1 to S8

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Fig. 1 (preceding page). Huntingtin and junctional complex proteins mislocalize in the ventricular zone of human fetuses carrying HD-causing mutations. (A) Left: Diagram showing the position of the fetal ventricular zone (VZ) relative to the cortical plate (CP). Right: Coronal brain sections of GW13 control human cortex were counterstained with DAPI. The dotted square shows the region imaged in (B). Scale bar, 100 μ m. (B) Left: Coronal GW13 brain sections from control fetus and fetus carrying HD-causing mutation (HD) were immunostained for HTT. Scale bars, 10 μ m. Right: Representative line-scan analysis (relative fluorescence intensity) of HTT immunostaining and quantification of apical/basal human HTT fluorescence intensity in the ventricular zone (for each condition, $n = 3$ fetuses from different mothers. Unpaired t test, *** $p = 0.0044$). (C and D) Coronal GW13 fetal brain sections were immunostained for ZO1 and PAR3 (C) and β -catenin and NCAD (D). Scale bars, 15 μ m. (E and F) Representative line-scan analysis (relative fluorescence intensity) of indicated immunostainings (top) and quantification of indicated fluorescence intensities in the ventricular zone (bottom graphs, for each condition, $n = 3$ fetuses from different mothers. ZO1: Unpaired t test, *** $p = 0.0003$; PAR3: Unpaired t test, * $p = 0.0177$; β -cat: Unpaired t test, *** $p = 0.0003$; NCAD: Mann-Whitney U test, $p = 0.4682$). ns, not significant. Results are mean $\pm$ s.e.m. VZ, ventricular zone; iSVZ, inner subventricular zone; oSVZ, outer subventricular zone; IZ, intermediate zone; CP, cortical plate. Nuclei were counterstained with DAPI.

Fig. 2 (next page). Junctional protein complexes are disrupted in the apical endfeet of HD mouse embryos. (A) Schematic of the *in utero* electroporation experiment. (B and C) Mouse embryos were electroporated at E13.5 with a pCAG-GFP construct to delineate the apical endfoot in E15.5 cortices. (B) Hdh^{Q7/Q7} and Hdh^{Q111/Q111} cortical sections were immunostained for GFP (left) and for HTT and GFP (right). White arrowheads point to apical endfeet. Nuclei were counterstained with DAPI. (C) Left: Diagram indicating the position of junctional complexes at the apical endfeet. Right: Cortical sections were immunostained for GFP, ZO1 and PAR3 (upper panel) and GFP, NCAD and β -Cat (lower panel). Scale bars, 5 μ m (B) and 2 μ m (C). (D) ZO1, PAR3, NCAD, β -catenin and vinculin immunoblotting analyses of lysates from E15.5 Hdh^{Q7/Q7} and Hdh^{Q111/Q111} cortices. Histogram corresponds to the quantitative evaluation of the indicated proteins (for each condition, $n =$ at least 7 embryos from different mothers. Unpaired t test. ZO1: ** $p = 0.0026$; PAR3: ** $p = 0.0075$; NCAD: $p = 0.1255$; β -cat: $p = 0.1476$). ns, not significant. Results are mean $\pm$ s.e.m. (E) HTT-associated complexes were immunoprecipitated with the 4C8 antibody from E15.5 Hdh^{Q7/Q7} and Hdh^{Q111/Q111} cortical extracts. Mouse IgG (mlgG) was used as a negative control.

Fig. 3 (preceding page). Interkinetic nuclear migration and mitosis of cortical APs are impaired in HD mouse embryos. (A) Schematic of the experiment for analysis of interkinetic nuclear migration. E13.5 Hdh^{Q7/Q7} and Hdh^{Q111/Q111} embryos were electroporated with Cdt1-mKO2 and Geminin-GFP constructs. After 48h, the movement of the nuclei, labeled with GFP and mKO2, was followed by spinning disc microscopy, taking one image every 10 or 15 min for 10 hours. (B to D) Representative images showing the movement of nuclei in G1, G2 and G1/S transition phases as indicated. (D) Stars indicate the beginning and ending of the G1/S transition. Scale bars, 5 μ m. (E) Quantitative differences in the velocities of G1 (for each condition: n = 9 cells from 3 embryos from different mothers. Unpaired *t* test: ****p* = 0.0008) and G2 phase nuclei (for each condition, n = at least 202 cells from 4 embryos from different mothers. Mann-Whitney U test: ****p* < 0.0001) and length of G1/S transition (for each condition, n = at least 8 cells from 3 embryos from different mothers. Unpaired *t* test: **p* = 0.0356). (F) Bar graphs show the percentage of phospho-histone 3 (PH3) cells (mitotic index) of dividing progenitors (E13.5: for each condition, n = at least 2151 cells from 4 embryos from different mothers. Unpaired *t* test: ****p* < 0.0001; E15.5: for each condition, n = at least 1801 cells from 3 embryos from different mothers. Unpaired *t* test: ****p* = 0.0005). Results are mean $\pm$ s.e.m.

Fig. 4 (next page). Mutant huntingtin shifts neurogenesis toward neuronal lineage. (A) Diagram showing the position of the fetal ventricular zone (VZ). (B) Cortical sections of GW13 fetuses were immunostained with antibody against phospho-histone 3 (PH3) and the mitotic index was quantified (for each condition, n = at least 1146 cells from 3 fetuses from different mothers. Mann-Whitney U test: ****p* < 0.0001). Scale bars, 25 μ m. (C) Coronal GW13 brain sections from control fetus and fetus carrying HD-causing mutation (HD) were immunostained for the cilia marker Arl13b. Scale bars, 5 μ m. Bar graphs show cilia length (for each condition, n = at least 770 cilia from 4 fetuses from different mothers. Mann-Whitney U test: ****p* < 0.0001) and cilia density (for each condition, n = 4 fetuses from different mothers. Unpaired *t* test: **p* = 0.0104) at the apical surface. (D) Coronal brain sections of GW13 human cortex were immunostained for F-actin, γ -tubulin (γ -tub) and Arl13b. Scale bars, 2 μ m. White arrowheads and white arrows show apical and baso-lateral cilia respectively. Bar graph shows the percentage of baso-lateral cilia at the apical surface (for each condition, n = at least 260 cilia from 4 fetuses from different mothers. Mann-Whitney U test: ****p* = 0.0003). (E) Typical PAX6 and TBR2 staining of a GW13 human fetal sample analyzed. Scale bars, 50 μ m. Bar graphs show the percentage of PAX6/TBR2 positive cells (PAX6⁺/TBR2⁺) over PAX6 positive TBR2 negative (PAX6⁺/TBR2⁻) progenitors (for each condition, 3 fetuses from different mothers were analyzed. VZ: n = at least 2447 cells. Mann-Whitney U test: ****p* < 0.0001; iSVZ: n = at least 1580 cells. Unpaired *t* test: **p* = 0.011). Results are mean $\pm$ s.e.m. VZ, ventricular zone; iSVZ, inner subventricular zone; oSVZ, outer subventricular zone. Nuclei were counterstained with DAPI.