Correction

NEUROSCIENCE

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Faulty neuronal determination and cell polarization are reverted by modulating HD early phenotypes

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Increasing evidence suggests that early neurodevelopmental defects in Huntington’s disease (HD) patients could contribute to the later adult neurodegenerative phenotype. Here, by using HD-derived induced pluripotent stem cell lines, we report that early telencephalic induction and late neural identity are affected in cortical and striatal populations. We show that a large CAG expansion causes complete failure of the neuro-ectodermal acquisition, while cells carrying shorter CAGs repeats show gross abnormalities in neural rosette formation as well as disrupted cytoarchitecture in cortical organoids. Gene-expression analysis showed that control organoid overlapped with mature human fetal cortical areas, while HD organoids correlated with the immature ventricular zone/subventricular zone. We also report that defects in neuroectoderm and rosette formation could be rescued by molecular and pharmacological approaches leading to a recovery of striatal identity. These results show that mutant huntingtin precludes normal neuronal fate acquisition and highlights a possible connection between mutant huntingtin and abnormal neural development in HD.

Huntington’s disease | neurodevelopment | striatal differentiation | organoids | human iPSC lines

Huntington’s disease (HD) is an inherited neurodegenerative disease caused by an expansion of CAG repeats in the huntingtin gene (HTT). While normal huntingtin (HTT) has a ubiquitous cellular localization and pleiotropic functions in the adult brain, mutant HTT (muHTT) appears to have a more selective pathogenic effect, as symptoms mostly relate to the loss of neurons within the striatum (1) and cortex (2). HTT also plays an essential role during early development in the organization of embryonic tissues, and complete loss-of-function of HTT leads to early embryonic death (3, 4). The fact that mutant HTT can, however, rescue lethality in HTT-KO embryos, indicates that the extraembryonic function of huntingtin does not depend on the length of the CAG tract (5), so that muHTT can rescue for loss of the wild-type form. Thus, different parts of the protein are involved in different functions during early development and late in life. Abnormal brain development has also been linked to the loss of HTT (6) and increasing evidence suggests a neurodevelopmental component in HD (7, 8). However, what remains poorly understood is whether the increased striatal and cortical neuronal vulnerability caused by muHTT is due to specific effects in the adult brain or if it is caused by abnormal development that leads to neurodegeneration in late life.

So far, the main evidence suggesting a neurodevelopmental component in HD is threefold. First, HTT interacts with a number of developmental factors (9, 10) and is implicated in several aspects of neural development, such as epiblast formation (11, 12), neural rosette and neural tube formation (13, 14), as well as neuronal survival and cortical maturation and migration (12, 15, 16). Second, there are predictions that the patho-


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Data deposition: Microarray data RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI, https://www.ebi.ac.uk/arrayexpress (accession no. E-MTAB-5964).

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neural rosette formation. Moreover, muHTT led to defects in cortical and striatal progenitor and neuronal specification, cytoarchitecture, and terminal neuronal maturation, with the HD lines showing an overall immature profile compared with control cultures and human embryonic brain tissue. Importantly, both zinc finger protein (ZFP) repressor directed against muHTT and a pharmacological approach by small molecule efficiently rescued early and late phenotypes in HD lines.

These data show that muHTT affects neuronal differentiation from early neuroectodermal fate acquisition to late striatal and cortical identity. Our data highlight a role for muHTT in neurodevelopment that could lead to the pathological phenotypes seen in HD patients in later life.

Materials and Methods

Human iPSC Cultures. Nonintegrating HD and control (CTR) iPSC lines were obtained from Mattis et al. (23). Cell lines were generated by reprograming using the Amaza Human Dermal Fibroblast Nucleofector Kit to express episomal plasmids, as previously reported (23). The lines/cloones used in this study were regularly tested and maintained mycoplasma-free. Cultures tested in this work are reported in Table S1. Karyotype for each cell line/clone was regularly monitored every 3 mo during the passages by Q-bandancing analyses (ISENET). Cells were maintained in mTeSR1 medium (Voden) and plated on Matrigel (Becton Dickinson). At 80% of confluence, iPSC colonies were mechanically isolated and transferred onto new plates.

Striatal Differentiation. Human HD and CTR iPSC lines were exposed to striatal differentiation protocol published in Delli Carri et al. (24). All differentiation experiments performed in this study have been conducted using cells from passage 20 to passage 50 (Table S1). Briefly, cells were plated at a density of 0.6 × 10^6 cells/cm^2 on Matrigel-coated dishes in Matrigel with 10 mM CaCl2, 1 mM MgCl2, 3 mM KCl, and 10 mM Hepes/NaOH (pH 7.4). The pipettes were filled with a solution composed of: 128 mM KCl, 10 mM NaCl, 11 mM EGTA, and 10 mM Hepes/KOH (pH 7.4). The patch pipette for the study of the biophysical properties of voltage-gated Na+ currents was filled with 120 mM NMDG, 10 mM CaCl2, 20 mM TEA-Cl, 10 mM EGTA, 2 mM MgCl2, 4 mM Mg-ATP, and 10 mM Hepes/CaSO4 (pH 7.4). The extracellular solution contained: 135 mM NaCl, 1 mM CaCl2, 2 mM MgSO4, 10 mM glucose, 5 mM tetraethylammonium-Cl, and 10 mM Hepes/NaOH (pH 7.4).

Immunocytochemistry. Cell cultures were fixed in 4% (vol/vol) paraformaldehyde for 15 min at room temperature at different time points analyzed. Subsequently, cells were blocked in 5% normal goat serum (NGS; Vector), 0.5% Triton X-100 in PBS at room temperature. Cells were then incubated overnight at 4 °C with primary antibodies diluted in solution containing 2.5% NGS and 0.25% Triton X-100. Images were acquired with a Leica DMI 6000B microscope (10× and 20× objectives) and analyzed with LAS-AF imaging software and then processed using ImageJ, only to adjust contrast for optimal RGB. Additional images were acquired with confocal microscopy (Molecular Probes) and then converted using Cell Profiler. The following primary antibodies were used: OCT4 (mouse, 1:100; Santa Cruz), PAX6 (rabbit, 1:300; Covance), SOX2 (rabbit 1:200; Millipore), PAX6 (mouse, 1:1,000; DHSB), FLAG (rabbit, 1:500; Sigma), N-cadherin (1:800; Becton Dickinson), PALSL1 (rabbit, 1:50; Santa Cruz), FOXG1 (rabbit, 1:500; Stemcell), GSX2 (rabbit, 1:500; Millipore), ASC1 (mouse, 1:500; Becton Dickinson), ISL1/2 (mouse, 1:500; Abcam), KIAA0320 (rabbit, 1:500), TBR1 (rabbit, 1:1,000; Promega), Map2a/b (mouse, 1:500; Becton Dickinson), CTIP2 (rat, 1:500; Abcam), DARPP32 (rabbit, 1:200; Abcam), TBR1 (rabbit, 1:1,000; Abcam), TBR2 (rabbit, 1:100; Abcam). Secondary antibodies conjugated to Alexa fluorophores 488 or 568 (Molecular Probe, Life Technologies) were used at a 1:1,000 dilution in PBS at room temperature for 1 h. Nuclei were visualized with Hoechst 33258 (5 μg ml^-1; Life Technologies).

Immunohistochemistry. Organoids were fixed in 4% (vol/vol) paraformaldehyde overnight at 4 °C. They were washed in PBS and transferred in a 30% sucrose solution at 4 °C for 48–72 h. Next, organoids were transferred into Tissue-Tek OCT compound, frozen immediately on dry ice and stored at −80 °C. Next, 15-μm-thick sections were generated using a cryostat. Cryosections were washed with PBS and blocked 10% NGS, 0.3% Triton X-100 in PBS at room temperature. Primary antibodies used were diluted in solution containing 5% NGS and 0.1% Triton X-100: NESTIN (mouse, 1:300; Millipore), K67 (rabbit, 1:500; Abcam), BLBP (rabbit, 1:500; Millipore), p-VIMENTIN (mouse, 1:100; Hybridoma Bank), SOX2 (rabbit, 1:200; Millipore), Map2a/b (mouse, 1:500; Becton Dickinson), TBR1 (rabbit, 1:1,000; Abcam), TBR2 (rabbit, 1:1,000; Abcam), CTIP2 (rat, 1:500; Abcam), Sema3G (rabbit, 1:500; Abcam). Secondary antibodies conjugated to Alexa fluorophores 488 or 568 (Molecular Probe, Life Technologies) were used 1:1,000 dilution in PBS at room temperature mixed with Hoechst 33258 (5 μg ml^-1; Life Technologies) to visualize nuclei.

ZFPs Cloning Strategy. The coding sequence for ZFP-A represor designed to repress muHTT was cut from the corresponding pVAX plasmid using EcoRI-XbaI. Instead, the ZFPΔDBD sequence was obtained by EcoRI-HindIII digestion from the plasmid pA6V-6PSEWB-ZFPΔDBD (Evotec) and blunted at the HindIII site. The sequences were then subcloned into the tetracycligregulated lentiviral plasmid FuW-tetO-MCS (Addgene #84000). Constructs were designed as bicistronic vectors carrying a puromycin resistance gene located downstream of the 3′ region of the ZFP coding sequence separated by a “self-cleaving” T2A peptide (Si Materials and Methods), allowing for enrichment of ZFP expressing cells upon induction with doxycycline and selection with puromycin. For the cloning of the ZFPs-T2A-puroR sequences into constitutive plasmid under the control of chicken β-actin promoter, both ZFPs-T2A-puroR were cut from the lentiviral constructs using EcoRI-Afel and ligated into the pCAG-Gre (Addgene #26647) recipient plasmid upon excision of Cre by EcoRI-Ntall digestion and 5′ blunting at the NotI site. Additional details are reported in Si Materials and Methods.

Real-Time qPCR. MRNA was isolated using TRIzol reagent according to the manufacturer’s instruction (Life Technologies). Potential contaminating DNA was removed by DNA-free kit (Ambion). Template cDNA was prepared by

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reverse transcription of 500 μg RNA using lScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using the CFX96 real-Time System (Bio-Rad). All reactions were performed in 15 μL containing 50 ng cDNA and SsoFast EVAGreen Supermix (Bio-Rad). Primer pairs used were reported in SI Materials and Methods.

Western Blot Analysis. Cell cultures were homogenized in RIPPA buffer (Tris-HCl pH 8 50 mM, NaCl 150 mM, SDS 0.1%, NP401%, Sodium deoxycholate 0.5%) supplemented with PMMS 1 mM, DTT 0.5 mM and protease inhibitor mixture (Sigma). Total protein was quantified using the BCA Kit (Thermo Scientific). Twenty micrograms of protein was loaded per track on a 7.5% SDS/PAGE gel for OCT4 and NANOGEN detection. Fifty micrograms of protein was loaded for MAP2a/b and DARPP32 per track on a 6% and 10% SDS/PAGE gels, respectively. Protein were transferred onto a nitrocelluose membrane and blocked in TBS-T with 5% nonfat dry milk (Bio-Rad). Nitrocelluose membranes were immunoprobed with OCT34 (mouse, 1:1,000; Santa Cruz), OCT4 1-14 aa (rabbit, 1:1,000; Abcam), OCT4 C-end (rabbit, 1:1,000; Abcam); NANOGEN (rabbit, 1:500; Abcam); MAP2a/b (mouse, 1:500; Becton Dickinson); DARPP32 (rabbit, 1:200; Abcam), N-CAD (mouse, 1:500; Becton Dickinson) at 4 °C overnight. Secondary antibody probing and detection was by use of the ECL Kit (Bio-Rad). α-Tubulin (1:10,000, mouse; Sigma) and GAPDH (1:3,000, mouse; Abcam) were used to normalize. The acquisitions were performed by Chemidoc MP (Bio-Rad) and densitometric analysis was performed by Image Lab software.

Microarray Analysis. To perform the microarray analysis, the Illumina HumanHT-12 v4 Expression BeadChip platform was used to perform differential expression analysis on DIV45 and DIV105 of differentiation of iPSC Q21n1 and Q109n1. A starting amount of 300 ng of high-quality total RNA was used to generate cDNA and cRNA with the Illumina TotalPrep RNA Amplification Kit (Illumina). The procedure consisted of RT with an oligo(dt) primer followed by a T7 promoter using Array-Script. The obtained cDNA became a template for in vitro transcription with T7 RNA polymerase and biotin UTP, which generated multiple copies of biotinylated cRNA. The purity and concentration of the cRNA were determined using a NanoDrop ND-1000 Spectrometer. Quality cRNA was subsequently hybridized using a direct hybridization array kit (Illumina). Each cRNA sample (1.5 μg) was hybridized overnight using the HumanHT-12v4 BeadChip array in a multiple-step procedure; the chips were washed, dried, and scanned on the BeadArray Reader (Illumina).

Data Processing and Pathway Analysis. Raw microarray data were generated using BeadStudio v3.0 (Illumina) and background-corrected for P < 0.01. Absolute expression values were log2-transformed and normalized using quantile normalization. Principal component analysis (PCA) was performed using the R package ade4 (27). Transcriptional profiles of CTR and HD organoids at DIV15 and DIV105 were compared with those of the developing human fetal brain using a machine-learning algorithm called CoNTExT that is trained on 1,340 primary tissue samples (28). In particular, we employed the laminar expression data dissected via laser-capture microdissection from the fetal human brain. To identify differentially expressed genes at DIV45, a t test was applied and only genes with P < 0.01 were considered significant. Gene Ontology (GO) analysis of genes with a higher expression in CTR organoids was performed using the ClueGo plugin of Cytoscape. The GO “biological component” was used to query these genes against the background of all genes expressed in the organoids. Only GO with a P < 0.01 were considered (k-score threshold = 0.4). The rest of the settings were left as defaults. To calculate the semantic similarity score between GO terms the R package GOSemSim was used (29). The similarity score between GO terms was subsequently transformed into clusters with the REVIGO webtool (30). Functional annotation of the genes was performed by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/). Microarray data RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession no. E-MTAB-5964. More detail is available in SI Materials and Methods.

Statistical Analysis. One-way ANOVA, Tukey posttest was used for all biologic experiments performed on more HD and CTR lines (*P < 0.05, **P < 0.01, ***P < 0.001).

Results

Large CAG Repeats in Huntington Gene Lead to Neuroectodermal Acquisition Defects. Integration-free HD and iPS cells were previously generated from fibroblasts of subjects carrying Q21, Q28, Q33, Q60, Q109, and Q180 (respectively with 21, 28, 33, 60, 109, and 180 CAG repeats) (Table S1) (23). Total HTT mRNA was similar among all iPS cell lines and clones (Fig. S1A).

With the aim of investigating the role of muHTT in the early phases of neural development, we exposed cells to a stepwise protocol that mimics human ventral telencephalon development (24, 31) (Fig. 1B).

We first compared the neurogenic potential of all CTR and HD-iPSC lines and clones by measuring their transition from pluripotency to neuroectodermal development to telencephalic specification, as judged by total numbers of OCT4*, PAX6*, and FOXG1 cells at early stages of differentiation. All iPS cell lines were pluripotent, as attested to by OCT4 and SOX2 staining performed at DIV0 (Fig. S1C). Following dual SMAD-inhibition, CTR lines Q21n1, Q28n4, and Q33n1 showed the expected PAX6* ectodermal acquisition and OCT4 down-regulation at DIV15 (Fig. 1A and B and Fig. S1D). In contrast, HD lines Q60n5, Q60n8, Q109n1, Q109n4, Q109n5, Q180n1, and Q180n3 showed that OCT4* cells were still present at the same time point (Fig. 1A–C and Fig. S1D). Remarkably, at day 8 there was a clear correlation (r = 0.97, P = 6.4e–15) between the number of OCT4* cells and the number of CAG repeats (Fig. 1D). To further check the validity of the OCT4 immunocytochemistry data, we performed Western blot and qPCR analyses on two or three, respectively, control lines and three HD lines (Q60n5, Q109n5, Q180n1), confirming that HD lines showed defects in OCT4 down-regulation following differentiation (Fig. S1 E and F). Interestingly, and contrary to this finding, NANOGEN was completely down-regulated upon differentiation in all CTR and HD lines (Fig. S1 G and H). Increased PAX6* cells and mRNA level were found during neuroectodermal differentiation in all three CTR and in Q60n5 lines, whereas Q109n1 and Q180n1 lines showed only slowest or modest increases during differentiation (Fig. 1A–C and Fig. S1D). In agreement with this high expression of OCT4 and low expression of PAX6, Q109n5 and Q180n1 lines, but not the lines with fewer CAG repeats, showed expression of mesodermal markers like α-smooth muscle actin (α-SMA) and α-fetoprotein (AFP) at a later stage in culture (32, 33) (Fig. S1 J-L).

These data indicate that CAG expansion in HTT gene specifically interferes with the down-regulation of the OCT4 pluri-potency gene, and with the proper acquisition of a PAX6* neuroectodermal fate.

Defective Ventral Telencephalic and Striatal Identity Acquisition in HD-Derived Neural Progenitors. Next, we tested the effect of muHTT at subsequent stages of the developmental program (i.e., in the emergence of neural rosettes and in the steps that precede neuronal specification). We focused on HD lines Q60n5 and Q109n1, because they show loss of pluripotency at day 15 and are therefore expected to progress properly toward differentiation (Fig. 1D). Fig. 2A shows that at DIV15 cultures of Q21n1 CTR were composed of typical N-CADHERIN* (N-CAD*) large rosettes made of polarized NESTIN* (NES*) neuroepithelial stem cells, radially aligned around a central lumen, whose mean size is of 216.4 ± 80.7 μm². In contrast, a significant reduction in lumen size was found in Q60n5 and Q109n1 lines (area of lumen size in square micrometers Q60n5: 80.13 ± 15.8, Q109n1: 87.1 ± 31.8) (Fig. 2A and B).

We then analyzed whether the early defects in neuroectodermal acquisition and rosette formation influence the ability of HD lines to acquire a ventral telencephalic fate, as

Student t test was used to compare only two groups (**P < 0.05) (Fig. 6 A and B and Figs. S4 B, C, L, and M and S5 C and D).
judged by FOXG1 expression, a transcription factor (TF) required for the development of the mammalian ventral telencephalon (31, 34). At DIV30 of differentiation, no differences in FOXG1 expression were found between CTR and HD lines, regardless of their genotype, indicating that the HD mutation does not interfere with the acquisition of proper telencephalic identity (Fig. 2 C and D).

We then looked at TF expression patterns to distinguish progenitor, immature, and mature neuronal populations coexisting within the human lateral ganglionic eminence (Fig. 2E). To begin with, we analyzed GSX2 expression, a key regional marker that identifies early striatal progenitors (31, 35, 36). Strikingly, we found that the percentage of GSX2+ cells and GSX2 mRNA levels were significantly reduced in HD lines compared with CTR lines (Q60n5: 30.22% ± 15%; Q109n1: 27.8% ± 3%, vs. CTR: 55.55 ± 11%) (Fig. 2 C, F, and G).

In vivo studies of human fetal development (31) demonstrate that the GSX2+ cells from the ventricular zone (VZ) migrate toward the subventricular zone (SVZ) while acquiring a new positional identity characterized, first, by a double-positive GSX2+/ASCL1+ state, followed by a GSX2+/ASC11+ state, before entering into the marginal zone (MZ) (Fig. 2E). We detected a 47% and 53% reduction in GSX2+/ASCL1+ cells and a 32% and 64.3% reduction in ASCL1+ cells, respectively, in Q60n5 and in Q109n1 lines compared with CTR (Fig. 2 C, H, and I), as well as lower ASCL1 expression in the Q109n1 line (Fig. 2J).

In the developing human striatum, the postmitotic progenitors migrate into the MZ and acquire CTIP2 expression (Fig. 2E) (31, 37). Interestingly, at DIV30 of differentiation the HD lines showed a marked reduction in the percentage of CTIP2 immunopositive cells (Fig. 2K). Compared with CTRs, Q60n5 and Q109n1 lines had a 25 ± 5.3% and 30 ± 2.6% drop in the percentage of CTIP2+ cells (Fig. 2L). Western blot and qPCR analysis confirmed the significant reduction in CTIP2 protein and mRNA expression in HD lines (Fig. 2 M–O). In addition, immunostaining and qPCR analysis revealed reduced levels of ISLT1 and NKX2.1, two TF involved in ventralization and expressed both in the SVZ and MZ, together with CTIP2 (31) (Fig. S2).

We next evaluated the capacity of the HD cells to complete neuronal maturation and generate striatal neurons. As expected, CTR lines were very efficient in their neuronal differentiation capacity, giving rise to dense clusters of βIII-tubulin+ (TUBB3+) and MAP2+ neurons. Conversely, a decrease in TUBB3+ and MAP2+ immunoreactive neurons was found in HD lines (Fig. 3A). Western blot analysis revealed that the decrease in MAP2a/b isoform levels in HD lines is inversely correlated to the CAGs expansion (Fig. 3 B and C). The presence of the 70-KDa MAP2c in the Q109n1 line, an isoform specifically found early in development, further confirmed the incomplete or delayed maturation of these cells (Fig. 3B). Finally, we analyzed the expression of markers for terminal striatal differentiation, like CTIP2 and DARPP32. At DIV50, coexpression of these two markers was observed both in CTR and, to a minor extent, in HD lines (Fig. 3D). Western blot and qPCR analysis for DARPP32 showed a clear reduction in protein and mRNA levels confirming a defect of the HD lines when undergoing terminal maturation into medium spiny neurons (MSN) (Fig. 3 D–F and Fig. S3A).

Next, we profiled neurons derived from CTR and HD lines for their electrophysiological properties by patch-clamp analysis at DIV30. A total number of 325 CTR cells, 180 Q60n5 cells, and 170 Q109n1 cells were registered for the different electrophysiological parameters. Voltage-gated Na+ inward currents evoked at different membrane potentials showed that 64% (n = 140) of CTR neurons, 51% (n = 73) of Q60n5 and 20% (n = 41) of Q109n1 displayed Na+ current peak larger than 500 pA, an arbitrary threshold defined to select in vitro mature stem cell-derived neurons (24) (Fig. 3G). In parallel, we found that 58% of CTRs, 41% of Q60n5, but only 8% of Q109n1 neurons displayed single spike or repetitive firing upon stimulation (Fig. 3H). Accordingly, the analysis of the Na+ current revealed that its density peak measured at −20 mV is higher in CTRs (89 ± 7 pA/pF; n = 78) than in Q60n5 (61 ± 10; n = 27) and in Q109n1 (51 ± 6; n = 17) (Fig. 3I). Interestingly, qPCR analysis for the levels of sodium channel transcripts SCN1B, SCN2B, SCN3B, and SCN4B, demonstrated a significant decrease in their expression in HD compared with CTRs (Fig. S3 B–E).

Overall, our data suggest that HD lines are defective in the acquisition of a GSX2+, ASCL1+, ISLT1+, NKX2.1+ ventral telencephalic progenitor identity and in striatal fate determination and terminal maturation.

MuHTT Expression Precludes Cortical Specification and Cell Compartmentalization. To investigate whether muHTT affects also human cortical neuronal specification, we differentiated one control (Q21n1) and one HD line (Q109n1) to cortical projection neuronal cultures (25). Immunostaining and quantification for cortical-specific markers revealed a significant reduction...
in the percentage of TBR2+ and TBR1+ cells in Q109n1-derived cortical cultures compared with Q21n1 line (Fig. S4 A and B). Moreover, qPCR analysis also showed a significant reduction in PAX6, TBR1, and TBR2 transcript levels in Q109n1 cells (Fig. S4C).

In addition, we exposed two CTR (Q21n1 and Q28n6) and two HD (Q60n5 and Q109n1) lines to a 3D differentiation protocol to obtain human cerebral organoids that more closely resemble HD (Q60n5 and Q109n1) lines to a 3D differentiation protocol (Fig. S4D). We observed that following neuroectodermal differentiation, CTR cells presented a radial NES+ and p-VIM+ neuroepithelium surrounding a fluid-filled cavity reminiscent of an embryonic ventricle, with polarized distribution of K67+ and qPKC+ staining at DIV60 (Fig. 4 A and B). Strikingly, these structures were completely absent in HD-derived corticoids. Moreover, NES and p-VIM staining showed an altered columnar organization with absence of K67+ cell polarity (Fig. 4 A and B). Accordingly, Sox2+ progenitor and MAP2+ neuronal populations clearly localized into two different population compartments in the CTR lines but failed to segregate in the HD lines at DIV85 (Fig. 4 B and Fig. S4E). In addition, Pax6, Tbr1, and Ctip2 expression and compartmentalization were also disrupted in HD samples (Fig. 4 B and Fig. S4E).

Collectively, these results reveal that mHTT severely affects cortical fate differentiation and proper cell organization in 3D models in vitro.

**HD Cerebral Organoids Show an Immature Transcriptional Blueprint.** To explain in detail the impairment observed both in cortical specification and in cell organization in HD cultures, we evaluated global transcriptional differences between CTR and HD organoids at DIV45 (n = 3 for each line) and DIV105 (n = 4 for CTR, n = 5 for HD). PCA showed a clear separation of the HD and CTR iPSC lines in the first principal component (PC1) at DIV45 and DIV105 (Fig. S4F). To assess the maturity reached by the CTR and HD organoids at these two time points, we
compared their transcriptional profiles to those of the transitioning in vivo laminae of the developing cortex using the machine-learning algorithm called CoNTExT (28). We found that Q21n1 DIV45 and DIV105 samples had the strongest overlap [max-log$_{10}$(P value) > 50] with the mature suplate zone (SP)/inner cortical plate (CPi)/outer cortical plate (CPo) and with the subpial granular

Fig. 3. Defective striatal terminal differentiation and maturation in HD lines. (A) Immunostaining for TUBB3/MAP2 at DIV30 of differentiation of Q60n5 and Q109n1 and CTR Q21n1. (Confocal images, scale bar, 50 μm.) (B) Representative Western blot for MAP2αβ at DIV30 with corresponding GAPDH levels. (C) Densitometric analysis on Western blot analysis normalized with GAPDH. (CTRs = 2.77 ± 0.74; Q60n5 = 1.61 ± 0.27; Q109n1 = 0.19 ± 0.04; *P < 0.05, **P < 0.001, *P < 0.05 one-way ANOVA; n = 3 biological experiments, data are represented as mean ± SEM.) (D) Double immunostaining for CTIP2 (red) and DARPP32 (green). (Scale bar, 100 μm; Inserts (crops of the same images), 50 μm.) (E) Representative Western blot for DARPP32 at DIV50 with corresponding GAPDH levels. (F) Densitometric analysis on Western blot analysis. (CTRs = 1.19 ± 0.21; Q60n5 = 0.85 ± 0.29; Q109n1 = 0.18 ± 0.09; *P < 0.05 one-way ANOVA; n = 3 biological experiments, data are represented as mean ± SEM.) (G) Families of Na$^+$ current traces evoked by the protocol (Lower traces) from iPSC-derived striatal neurons differentiated in vitro for 30 d. (H) The graph represents the percentage of cells able to generate 1 spike or firing at DIV30 of differentiation. 62% of CTRs, 53% of Q60n5, and only 8% of Q109 when activated displayed single spike or repetitive firing. (*P < 0.05 one-way ANOVA; n = 3 biological experiments, data are represented as mean ± SEM.) (I) The Na$^+$ current density recorded at −20 mV from the different iPSC-derived neurons. In the graph, each column represents the average of Na$^+$ current density in recorded cells. (Q21n1 plus Q33n1 n = 78; Q60 n = 27, Q109 n = 17, *P < 0.05 one-way ANOVA; n = 3 biological experiments, data are represented as mean ± SEM.)

Fig. 4. HD lines show defects in cortical differentiation and cytoarchitecture. (A) Immunocytochemistry for αPKC, NESTIN/Ki67 on organoids derived from Q21n1 and Q109n1. (Scale bar, 100 μm.) These images are representative of five organoids stained for each line generated in two independent experiments. Representative confocal images of organoids stained with NESTIN, Ki67, and p-VIMENTIN. (Scale bar, 25 μm.) (B) Organoids derived from Q21n1 and Q109n1, double-stained for SOX2/MAP2. (Scale bar, 100 μm.) Specific cortical markers PAX6/TBR1 and TBR1/CTIP2. Arrows indicate the structure mimicking the embryonic ventricle in Q21n1 organoids. Dotted ovals show SOX2 and MAP2 organization in Q21n1 and Q109n1. (Scale bar, 50 μm.)

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layer (SG), respectively (Fig. 5A, Left). In contrast, Q109n1 samples had the strongest overlap [max-log_{10}(P value) > 29] with the VZ/SP at DIV45 and with the SP at DIV105 (Fig. 5A, Right). This result suggests that HD organoids appear to remain “trapped” in the VZ/SP transitions, while CTR organoids mature to more mature entities. In line, immunocytochemistry of the organoids show that HD lines have a lower expression of TBR1 and CTIP2 that are present in the CTR line (Fig. 4B and Fig. S4E). This confirms the differences in maturity reached by the CTR line also at the protein level.

Subsequently, we proceeded in analyzing the differences in expression at DIV45 to determine early-stage transcriptional divergences and identified 1,452 differentially expressed genes (P < 0.01, t test) between the two groups; 685 genes had a higher expression in Q21n1 organoids and 767 had a higher expression in the Q109n1 line (Fig. 5B and Dataset S1). To further understand the biological relevance of the genes that are down-regulated in HD and that may explain the phenotype observed, we carried out a GO analysis using the clueGO plugin of Cytoscape (39). We then calculated the semantic similarity scores of all GO-term pairs (P < 0.01) and found a high enrichment for positive regulation of neuron projection development and chemical synaptic transmission (Fig. 5C), suggesting an immature transcriptional signature in HD organoids. We then searched for genes whose expression could be strictly related to muHTT effects. To do so, and to avoid noise due to the different background of the HD-iPSC lines, we compared the genes differentially expressed at DIV45 with the ones determined in another study comparing transcriptional differences between HD isogenic lines at the neural progenitor stage (22). We identified 381 genes differentially expressed between CTR and HD lines in common for both studies (Fig. S4G and Dataset S2) and submitted them to an IPA. Genes with a higher expression in HD organoids were significantly associated (P = 2.17E-07) with the main upstream regulator TGF-β, as shown by Ring et al. (22), whereas low expressed genes were significantly (P < 7.18E-04) associated with nervous system development, especially morphology of the cerebral cortex (Fig. S4H, Left, network with the highest-scored functional pathways; Right, heatmap showing genes enriched for these pathways). SOX11, GAP43, and CELSR3, all transcripts involved in neural migration and differentiation, were also down-regulated in HD cells (Fig. S4 I–K), and in E16.5 cortex from the HD mouse model R6/2 compared with control littermates (Fig. S4 L–N).

Overall, these data indicate that muHTT affects striatal and cortical cell specification and further neuronal maturation.

**Allele-Specific Down-Regulation of muHTT Rescues Neural Induction Defects in HD Lines.** We reasoned that specific down-regulation of muHTT at early stages of differentiation might reduce or reverse its adverse effect on neural induction. To confirm this hypothesis, we used a synthetic ZFP (provided by Sangamo) shown to efficiently work as selective repressor of muHTT. To this aim, a doxycycline-inducible ZFP type A (FLAG-tagged) protein was stably expressed in the Q109n5 line, to obtain time-controlled expression of the muHTT repressor. In this assay we used Q109n5 cell lines due to the persistent presence of OCT4+ cells during neural induction (Fig. 1 A–C). As a control, an inducible Q109n5 stable cell line expressing a DNA binding-domain mutant inactive version of the ZFP was also generated (ZFPΔDBD).

ZFP-A and ZFPΔDBD expressing Q109n5 cell lines were exposed to neural induction and analyzed for OCT4 expression at DIV5 (Fig. S5 A and B). Robust induction of ZFP-A and
ZFPΔDBD expression upon 5 d of doxycycline treatment was confirmed by qPCR (Fig. S5C). As expected, ZFP-A expression induced a down-regulation of total HTT mRNA that was not observed for control ZFPΔDBD (Fig. 6A). Interestingly, ZFP-A expression as judged by anti-FLAG staining, showed a significant reversion of the incomplete neuroectodermal transition observed for this line, with a 55% drop in the percentage of OCT4+ cells (Fig. 6B and Fig. S5B).

These data show that the defects observed during the pluripotency-to-neuroectodermal transition are directly dependent on expression of muHTT, and that selective down-regulation of muHTT effectively restores normal frequency of OCT4+ cells in vitro.

We then decided to investigate whether the early and continuous down-regulation of muHTT could have an impact at later maturation stages, on neural rosette formation. To achieve a robust and continuous silencing of muHTT, we decided to move the ZFPs into a constitutive plasmid under the control of the chicken 
β-actin promoter. Q21n1 and Q109n1 stable cell lines with constitutive expression of the ZFPs showed ZFP expression and HTT down-regulation at DIV8 (Fig. 6C and Fig. S5D). Rosette lumen size was evaluated by immunocytochemistry for PALS1 and N-cadherin in Q109n1 and control cultures expressing ZFPΔDBD and ZFP-A (Fig. 6D). Quantification of rosettes stained for PALS1 showed a significant rescue of 52.5% in lumen size in Q109n1 ZFP-A compared with cells expressing control ZFPΔDBD (Fig. 6E).
Altogether, these data demonstrate that transcriptional silencing of muHTT can have a significant impact in early developmental HD phenotypes.

**Modulation of muHTT Levels During Early Patterning Rescues Later Striatal Specification Defects.** In parallel with ZFP strategy, we decided to adopt a pharmacological approach by small molecule to inhibit muHTT deleterious effect from neural rosette stage (Fig. 2) to later stages in culture (DIV30). Our previous studies showed defects in rosette formation in HTT KO mouse ES cells, due to hyperactive metalloprotease ADAM10 and increased N-CAD cleavage, as revealed by an elevated N-CAD C-terminal fragment (CTF) by Western blot (14). We show here that the presence of muHTT in the Q109n1 line at DIV15 of differentiation leads to a similar increase in the CTF fragment (Fig. 6 F and G). The G1254023X compound is a selective inhibitor of ADAM10 activity that is able to restore neural tube defects and N-CAD cleavage in httMO-Zebrafish embryos (14). Importantly, exposure to 10 nM G1254023X from DIV5 to DIV8 of differentiation caused a significant reduction in the level of the CTF fragment, leading to a significant recovery of 54.2% in lumen size of Q109n1 cells (Fig. 6 F–I). To understand if the rescue in this early developmental phenotype influences late maturation of the HD cells at DIV30, we evaluated mRNA levels of general and striatal specific progenitor markers, such as SOX11, Gsx2, and CTIP2. qPCR for these markers performed at DIV30 of differentiation showed a 50% increase of SOX11 mRNA and 30% increase in Gsx2 and CTIP2 transcript levels upon G1254023X treatment (Fig. 6 J–L). Accordingly, the percentage of CTIP2+ cells was partially recovered in G1254023X treated Q109n1, compared with untreated cells (Fig. 6 M and N), and this is accompanied by an overall better neuronal differentiation as judged by Map2/TUBB3 staining (Fig. S5E).

Taken together, these results demonstrate that muHTT negatively affects striatal and cortical specification, differentiation, and maturation in vitro. This impairment is evident in both 2D monolayer neuronal cultures and 3D organoids showing an abnormal neuroectodermal transition associated with altered cortico/striatal specification, together with disrupted cellular organization. Strikingly, defects in 2D neuronal induction and striatal differentiation are successfully rescued by down-regulation of muHTT or pharmacological inhibition of one of its effectors, ADAM10, from the earliest step of the differentiation.

**Discussion**

HTT protein contributes to several cellular pathways during embryonic development and adulthood. Wild-type HTT is needed for normal development and its deletion in mouse causes embryonic lethality at E8.5, with severe abnormalities in neuronal development (40, 41). Mounting evidence suggests that muHTT could tamper with neuronal development with repercussions during adulthood that contribute to the HD pathology (42). However, there is no information as to how an early developmental defect may affect neuronal function in adulthood or whether early therapeutic intervention could have a significant disease modifying effect.

By using nonintegrating human HD-iPSC lines, we demonstrated that the pathologic expansion of the CAG tract affects neural cell positional identity, 3D self-organizing capacity, and terminal maturation. Problems in reaching a mature transcriptional profile by HD lines was also shown by the HD iPSC Consortium that revealed that the omics signatures of HD iPSC lines correspond to an earlier stage of mouse development compared with controls (8). In our study, HD lines showed reduced efficiency in ventral specification with reduced Gsx2, CTIP2, and MAP2 expression compared with controls. Moreover, HD lines manifest a delayed exit from pluripotency (Q60 and Q109 lines) that is further exacerbated in highly expanded CAG repeats (Q180 lines), where cells remained OCT4+ and committed to mesodermal differentiation even after neural induction. This is not surprising, considering that overexpression of PAX6 does not induce meso-endodermal fate while PAX6 knockdown does not inhibit meso-endodermal differentiation (43).

These data also call for a more detailed study on HTT sub-cellular localization during differentiation and the use of iPSC lines to reveal potential aberrant interactions between specific transcription factors and the mutant protein.

In addition to the striatal component, clear alterations in cortical fate were detected, with reduction of TBR2 and TBR1 levels in HD progenitors compared with controls. Accordingly, we reveal that polyQ-expanded HTT has a role in cell-to-cell interaction and tissue organization during cortical development because defects in neuronal positioning and cell organization were found in 3D organoids generated from HD iPSCs. These results, together with those from Barnat et al. and others showing that HTT removal in postmitotic projection neurons leads to defects in neuronal migration in the mouse neocortex, provide further evidence that HTT loss-of-function mechanisms associated with the pathologic expansion can operate during neural development (40–42, 44). Our HD organoids also revealed a transcriptional profile characterized by reduced expression of genes with key roles in neuronal migration and differentiation. Among them, SOX11, GAP43, and CELSR3 were downregulated also during 2D MSN differentiation, thus suggesting a common effect of muHTT during cortical and striatal differentiation, which contribute to explaining the selective vulnerability observed in the striatum and cortex of HD patients. This hypothesis was further sustained by the down-regulation of these transcripts in the brain of R6/2 embryos.

Importantly, studies of prodromal HD individuals revealed brain changes decades before the onset of the disease, including smaller head circumference (45), caudate and putamen atrophy (46, 47), and striatal and cortical white matter abnormalities (48), including cortical thinning (47).

Our results suggest that both the striatal and cortical signatures are flawed during development, with regards to the response to differentiation cues and ability to self-organize in complex 3D structures. We also show that the progression through subsequent steps of the developmental program is affected in a CAG-dependent manner, with the highest expansion limiting exit from pluripotency, Q109 and Q60, causing a delay in neuroectoderm formation, followed by the inability (greater in the Q109 line) to undergo full commitment to striatal specification.

Furthermore, our data attest that the defects in the transition from pluripotency to neuroectoderm can be reverted by down-regulating muHTT, and that interventions aimed at restoring defects in neural rosette formation are able to significantly revert later ventral specification abnormalities.

One major question that arises from these results is whether these developmental defects may be relevant to the in vivo pathology. Smaller intracranial volumes in presymptomatic HD patients have been observed in one study (44); however, this was not replicated in the TRACK-HD cohort (49). Whether the iPSC models used here correctly mimic what happens in HD still need to be investigated. In general, iPSCs have been widely used to model genetically encoded pathologies in vitro. In some cases, these cells have shown remarkably good recapitulation of disease-specific features. This was the case for Ebert et al. (50), where SMA patient-derived iPSCs differentiated to motor neurons showed survival and drug responses also found in patients. Also, Koch et al. (51) successfully used iPSCs to study Ataxin-3 aggregation in neurons from patients with Machado-Joseph disease. However, in some cases, iPSCs revealed subtle cellular characteristics that were not recapitulated (or not detected) in the patient. For example, long QT syndrome (LQT2) hiPSC-derived cardiomyocytes generated from a mutation carrier with normal QT, showed normal QT interval that was 170% longer.
that of controls (52). This phenotype was only observed in the iPS-derived cells, while the patient was asymptomatic. This leads us to conclude that in vitro cell modeling could in some cases exacerbate disease-related conditions that are masked in vivo by unknown compensatory mechanisms. Thus, later symptomatic changes in the HD brain could arise from subtle changes during neurodevelopment that are in some way “camouflaged” or compensated for, in a more complex in vivo environment. However, if these small alterations were present, they may change the regular neuronal homeostasis and in the long run lead to the symptoms observed in HD. To clear how relevant iPSCs are for HD modeling needs further examination.

In conclusion, these results advocate for the theory of HD as a pathology with a disguised neurodevelopmental component, where the polyQ expansion alters the canonical developmental path of striatal and cortical neurons that are therefore more unstable and susceptible to muHTT during adulthood. Given the clinical implication of such findings, conclusive in vivo evidence in humans remains mandatory.

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