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Blanca Diaz-Castro[1], Mohitkumar R. Gangwani[1], Xinzhu Yu[1], Giovanni Coppola[3,4,5] & Baljit S. Khakh[1,2*]

[1]Department of Physiology
[2]Department of Neurobiology
[3]Department of Neurology
[4]Department of Psychiatry and Biobehavioral Sciences
[5]Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine, University of California Los Angeles, Los Angeles USA CA 90095-1751

*To whom correspondence should be addressed: bkhakh@mednet.ucla.edu

One sentence summary: Astrocyte gene expression is altered in mouse models of Huntington's disease and in postmortem brain samples from HD patients.

Abstract

Astrocytes are implicated in neurodegenerative disorders and may contribute to striatal neuron loss or dysfunction in Huntington's disease (HD). Here, we assessed striatal astrocyte gene and protein signatures in two HD mouse models at three stages, and compared our results to human HD data at four clinical grades and to mice exhibiting polyglutamine-length dependent pathology. We found disease-model and stage-specific alterations and discovered a core disease-associated astrocyte molecular signature comprising 62 genes that were conserved between mice and humans. Our results show little evidence of neurotoxic A1 astrocytes that have been proposed to be causal for neuronal death in neurodegenerative disorders such as HD. Furthermore, 61 of the 62-core gene expression changes within astrocytes were reversed in a HD mouse model by lowering astrocyte mutant huntingtin protein (mHTT) expression using zinc finger protein (ZFP) transcriptional repressors. Our findings indicate HD astrocytes progressively lose essential normal functions, some of which can be remedied by lowering mHTT. The data have implications for neurodegenerative disease rescue and repair strategies, specific therapeutic relevance for mHTT reduction and contribute to a better understanding of fundamental astrocyte biology and its contributions to disease.

Introduction

Astrocytes closely contact neurons, blood vessels as well as other glia, and are widely implicated in CNS disorders, but their precise contributions remain incompletely understood (*1, 2*). We address this topic with a focus on Huntington's disease (HD) – a progressive neurodegenerative disorder characterized by motor, cognitive, and psychiatric symptoms (*3, 4*). HD is caused by an autosomal dominant mutation in the *HTT* gene (*3, 4*). Exon 1 of *HTT* contains a CAG trinucleotide repeat. Healthy subjects carry < 35 CAG repeats, but the disease is 100% penetrant when the number of CAG repeats exceeds 40. The average age of onset is ~45 years, and there is an inverse correlation between the CAG repeat length and the age of onset (*5*). Although the protein encoded by *HTT,* huntingtin (HTT), is expressed throughout the body, HD mainly affects the CNS and in particular the striatum and cortico-striatal-thalamic circuitry (*6*).

Selective expression of mutant huntingtin (mHTT) in astrocytes led to phenotypes related to HD in mice (*7*), and mHTT deletion from astrocytes in a HD mouse model slowed disease progression (*8*). Engraftment of mHTT expressing human glia caused HD-related phenotypes in mice, whereas engraftment of normal human glial cells in a mouse model of HD ameliorated the disease phenotype (*9*). In terms of mechanisms, astrocytes have been shown to display a variety of alterations in HD mouse models (*6*). Astrocytes from postmortem HD tissue contain mHTT, and display disease progression-dependent increases in GFAP expression associated with astrocyte reactivity (*6*). In mouse models, HD phenotypes occur in the absence of overt reactivity (*6, 10*), but strong GFAP expression is seen in lentiviral models (*11*). In relation to reactivity and its contributions to pathophysiology, it has been recently proposed that astrocyte reactivity comprises two broadly defined populations termed A1 and A2. Of these, A1 is proposed to be neurotoxic and is proposed to cause neuronal death in neurodegenerative diseases including HD (*12*). In the present study, we used systems biology and agnostic assessments of astrocyte gene and protein

expression in striatal tissue from mouse models and from postmortem human HD samples. We sought to identify core astrocyte molecular signatures associated with HD during disease progression in mice and humans, and the effects of astrocytic mHTT lowering in mice with zinc finger protein transcriptional repressors (*13*).

Results

Little evidence for neurotoxic A1 astrocyte signatures in HD

Astrocytes from HD model mice display a variety of known molecular and cellular alterations relative to controls (*6*). In order to evaluate additional alterations in an unbiased and comprehensive manner and to determine the relevance of the findings from mouse models, we assessed gene expression from human caudate HD specimens compared to healthy controls (**Fig. 1A**) (*14*). We compared these data to those from transgenic R6/2 and knock-in Q175 mouse models at three ages, and from an allelic series of knock-in mice harboring mHTT alleles of differing CAG lengths (**Fig. 1B**) (*15*). All assessments were relative to age-matched controls (**Fig. 1B**) and the human data were separated with neuroanatomical grade (**Fig. 1A,** fig. S1A). We evaluated 38 markers proposed to identify A1 and A2 reactive astrocytes, and microglial genes proposed to cause A1 astrocytes (*12, 16*).

Using a false discovery rate (FDR) < 0.05, several genes from pan reactive, A1 and A2 groups were upregulated in advanced stage human HD samples (**Fig. 1C**). With a $\log_2$ ratio greater than 2, upregulation was only observed at disease grade 3 for two of the reactivity genes (**Fig. 1C:** *CP, SERPINA3*). There were no consistent changes at grades 0 and 1 (**Fig. 1C**; n = 3-16 HD human samples with 32 controls). Around ~50% of caudate neurons are lost at grade 1 (*17*), which thus occurs without A1 signatures (**Fig. 1C**). The genes upregulated at grades 2 and 3 also did not fall into A1- or A2-specific categories (**Fig. 1C**). Similarly, when we evaluated microglia genes

thought to drive astrocyte reactive phenotypes, we observed $\log_2$ ratio changes > 2 only at grade 3 (**Fig. 1D**). However, of the striatal astrocyte enriched genes (*18*) around 20% were altered at grade 1, rising to ~50% at grades 2 and 3 (**Fig. 1C**). These analyses from human tissue indicate that marked astrocyte reactivity occurs at stage 3, but that additional astrocyte genes are altered at grade 1 (**Fig. 1C**).

We next assessed astrocytes in striatum of two HD mouse models using qPCR of 13 genes from the pan reactive, A1 and A2 classes (**Fig. 1E**; n = 4-7 mice each). One is a severe transgenic model (R6/2) that likely reflects juvenile onset HD (*10*). The other is a milder knock-in model (Q175), which develops slowly, reflecting adult onset HD (*19*). Neuronal loss is variable in the HD mouse models, although they display striatal volume loss and functional changes consistent with the human disease (*10, 20*).We assessed each mouse model in relation to controls, referred to as wild type (WT) for Q175 and non-carriers (NCAR) for R6/2. We explored presymptomatic (2 months for Q175, 1 month for R6/2), symptomatic (6 months for Q175 and 2 months for R6/2), and late symptomatic stages (12 months for Q175 and 3 months for R6/2).

Of the A1 specific genes evaluated by qPCR, only *Serping1* was consistently upregulated across models. (**Fig. 1E**). Clear evidence for reactivity was seen in LPS injected mice that we used as a control, but the changes were also not A1 specific either (**Fig. 1E**; n = 3-4). Next, we sought to explore upregulation of astrocyte reactivity genes by examining differential expression in the HD allelic series mice with different CAG repeat lengths (Q80, Q92, Q111, Q140 or Q175) relative to Q20 controls at matching ages of 2, 6 and 10 months (fig. S1B). Consistent with the human, R6/2 and Q175 data, we found no evidence of strong astrocyte reactivity or uniquely A1 signatures (**Fig. 1F,G**). We explored these observations at the protein level. GFAP immunohistochemistry (IHC) was performed for Q175 and R6/2 mice at three ages (**Fig. 1H, K**; n = 6). We quantified average integrated GFAP expression and found increases in Q175 vs WT mice at 12 months (**Fig.**

1H,I). Furthermore, upregulation of GFAP signal was only observed in R6/2 mice at 3 months relative to NCARs (**Fig. 1K,L**; n = 6-8). Using GFAP immunoblotting, an increase was detected at 12 months in Q175 vs WT, but not at earlier stages in Q175 or at any age in R6/2 vs NCAR (**Fig. 1J, M**; n = 3-6). We have no completely satisfying explanation for why GFAP in R6/2 mice increased relative to NCARs at 3 months when assessed by IHC and not when assessed by immunoblotting. Irrespectively, by this measure, only subtle reactivity occurred in Q175 and R6/2 models at the oldest ages (**Fig. 1**). The complement-associated gene *C3* was identified as one of the most highly upregulated A1 genes (*12*), but it was not differentially expressed in the HD mouse models studied here (**Fig. 1F**) and when evaluated by immunohistochemistry, C3 was only increased in R6/2 mice at 3 months (fig. S1C-F), which recalls human data (**Fig. 1C**). We assessed microglia activation using IHC for IBA1 and CD68 and did not observe increased immunostaining in HD models (fig. S1G-P; n = 3-6).

HD-related astrocyte differential gene expression, pathways and WGCNA

To identify astrocyte molecular changes in HD mouse models, we performed RNA-seq in the dorsolateral (d.l.) striatum of R6/2 and Q175 mice at multiple ages (**Fig. 2A**). Striatal astrocyte-specific RNA purification was achieved by using the AAV 2/5 Rpl22-HA RiboTag method (*21*) (**Fig. 2A** & fig. S2). Rpl22-HA expression was co-localized with astrocytes, but not with neurons (**Fig. 2B-E**; n = 3). Furthermore, the astrocyte-specific immunoprecipitated (IP) samples were replete with known astrocyte markers (for example, *Aldh1l1*, *Aldoc*, *Slc1a2*, *Gja1*), but depleted of other cell markers (**Fig. 2F-I**).

Astrocyte transcriptional differences in Q175 mice at 2 month, 6 month and 12 month, and in R6/2 mice at 1 month, 2 month and 3 month were evaluated (relative to controls) with principal component analysis (PCA; **Fig. 2J,K**). HD sample segregation from controls increased with age,

and the transcriptional differences associated with disease were confirmed by differentially expressed gene (DEG) analysis for IP and input RNA (fig. S3A,B; FDR < 0.05). Data for all the IP DEGs with a FDR < 0.05 are presented in fig. S4. We performed a range of analyses including the effect of different thresholds (fig. S5), astrocyte reactivity analyses (fig. S6), known gene families (fig. S7), candidate gene pathways (fig. S8-9) and Weighted Gene Co-expression Network Analysis (WGCNA) to identify genes and pathways with progressive changes correlated with disease severity (fig. S10-11). In relation to A1 reactive astrocyte signatures, there were qualitative differences between qPCR and RNA-seq data across A1 genes. For example, *H2-d1* was subtly upregulated by qPCR, but was downregulated in the RNA-seq data (**Fig. 1E**, fig. S6). To summarize these evaluations (fig. S5-11), we found no evidence for A1 reactivity, but we confirmed known alterations of K^+ channels, neurotransmitter transporters and calcium signaling, and identified hitherto unknown genes and pathways altered within astrocytes in a disease progression-dependent manner in HD.

Top 50 HD-related astrocyte DEGs

We focused on the top 50 down- and upregulated genes (FPKM > 10), representing the most pronounced differences in astrocytes from HD samples at each disease stage. The overlap between the top 50 changes across ages is summarized in **Fig. 3A-C** and displayed in **Fig. 3D-G**. The genes that were differentially expressed at a particular age, are marked with an * if they were within the top 50, or a # if they were below the top 50 (**Fig. 3D-G**). The arrows indicate astrocyte enrichment (2-fold cut off). Furthermore, in **Fig. 3D-G**, the genes from mouse models that changed in the human data are highlighted in green. For the Q175 mice, the concordance with human data was 89% for the downregulated genes and 49% for the upregulated ones (**Fig. 3D,E**). In the case of the R6/2 mice, the concordance with human data was 41% for the downregulated and also 41%

for the upregulated genes (**Fig. 3F,G**). The putative functions performed by the proteins encoded by the top 50 down or upregulated genes are summarized in **Fig. 3H**. The data reported in **Fig. 3** provide unbiased measures of astrocyte-selective in vivo gene expression changes from mouse models and humans for a neurodegenerative disease. Overall, the top astrocyte DEGs showed strong overlap between models and ages (**Fig. 3A-C**), and the data were consistent with changes in human samples (for the top 50, the changes consistent with human data are shown in green in **Fig. 3D-G**). The functions that these DEGs underlie are similar between early and late stages, suggesting that astrocyte pathways become dysfunctional early and continue to be affected, as shown in the plots reported in Fig. 3H.

Fig. S12 provides additional analyses for the shared genes across mouse symptomatic ages, including the numbers of genes (fig. S12A,B) and the identities for the top 50 up- and down-regulated across all four symptomatic data sets for R6/2 and Q175 mice (fig. S12C,D). In addition, fig. S12C,D also shows the genes that were up- and downregulated in the human data (green text) in accord with the changes observed in HD model mice. The functions of many of the identified genes are unknown. For example, *Adcy5* encodes an adenylate cyclase that is highly expressed in the striatum with known naturally occurring mutations associated with chorea and dystonia (*22*). Furthermore, *Srebf1*, which encodes for a transcription factor that regulates lipid homeostasis, is upregulated in human HD (*14*), and astrocyte enriched *Maoa* (*23*) carries polymorphisms identified as modifiers for cognitive symptoms in HD. Within the 126 common genes, *Mlc1* (fourth on the upregulated list) is astrocyte-enriched and displays the highest RNA expression. We validated its expression changes in HD models (fig. S12E-H; n = 9 slices from 3 mice). Mutations of *Mlc1* produce a recessive hereditary neurological disorder called megalencephalic leukoencephalopathy with subcortical cysts that causes macrocephaly, myelinopathy, deterioration of motor functions, ataxia and mental decline (*24*). Of the astrocyte genes that may account for

astrocyte contributions to HD, several are involved in important aspects of biology such as ion homeostasis, metabolism or cell morphology, whereas others have undefined roles (fig. S12C,D; **Fig. 3H**). Irrespectively, there was strong concordance in the top 50 DEGs between mouse models and human data (**Fig. 3D-G**, green text) providing a valuable resource for future hypothesis-driven experiments.

Astrocyte gene expression changes across mouse models and in humans

We used gene expression as well as proteomics from R6/2 and Q175 mice, and human HD samples to identify astrocyte molecules that were changed similarly (**Fig. 1B, 4A-G**), in other words those genes that were up- or downregulated across these samples in accordance with the astrocyte RNA-seq data. We observed strong agreement (>50%) between our common DEGs across four symptomatic datasets (6 and 12 month Q175; 2 and 3 month R6/2) with proteomic, as well as the human gene expression data (the numbers are reported in **Fig. 4D,G**). Overall, 62 DEGs were conserved across RNA and protein from HD mouse models and human postmortem RNA (**Fig. 4H**). The majority of these 62 genes were downregulated in HD samples in mouse and human (**Fig. 4I**). We further validated this dataset by checking if these genes were also differentially expressed in the allelic series at the RNA and protein level (**Fig. 4J,** fig. S13) and found that indeed this was the case. Moreover, they displayed greater agreement for the models with the highest CAG repeats (Q111 to Q175 in **Fig. 4J**), which provides support that the molecular changes track disease severity. Within this set of 62-core changes, many genes were involved in calcium-dependent processes (*Camk4*, *Prkcb*, *Atp2b1*, *Itpr1*, *Ppp3ca*, *Cacna1e*, *Ryr3*, *Atp2b2*, *Cacna2d3*, and *Ppp3r1*), GPCR (*Adcy5*, *Rgs9*, *Prkcb*, *Itpr1*, *Ppp3ca*, *Cacna1e*, *Plcb1*, *Gng7*, *Cacna2d3*, and *Ppp3r1*), and glutamate receptor signaling (*Rgs9*, *Shank3*, *Neto1*, *Cx3cl1*, and *Dlg4*). Within the 62 DEGs, the two astrocyte enriched upregulated genes were *Psat1* and *Rdx*.

Psat1 encodes an enzyme involved in L-serine production. L-serine is the precursor of D-serine, a neuromodulator for *N*-methyl-D-aspartate (NMDA) receptor, which may contribute to excitotoxicity (*20, 25*). *Rdx* encodes for radixin that via ezrin and moesin forms a complex between the cytoskeleton and plasma membrane. Changes in this gene may conceivably contribute to the reduction in size of HD astrocytes (*26*).

mHTT lowering in astrocytes with ZFPs

Astrocytes are proposed to contribute to disease via cell-autonomous and non-cell autonomous mechanisms (*27*). However, such mechanisms have not been systematically explored for HD. Based on recent work (*13*), we created AAVs to express ZFP transcriptional repressors to reduce mutant HTT (mHTT) within astrocytes. As a control, we used non-binding ZFPs (ZFPDelta). All comparisons were between ZFP and ZFPDelta groups to control for any potential effects of the AAV microinjections. To evaluate specificity we injected the ZFP and ZFPDelta AAVs into the d.l. striatum of WT mice and performed IHC (**Fig. 5A-C**). The expression of both ZFP and ZFPDelta strongly co-localized with S100β, an astrocytic marker, but not with the neuronal marker NeuN (**Fig. 5B,C**).

We next injected ZFP and ZFPDelta AAVs into the d.l. striatum of 4 week old R6/2 mice and after seven weeks analyzed mHTT (**Fig. 5D-J**) in five batches of mice (each batch comprised 3-4 mice). A ~60% reduction of mHTT inclusions in S100β positive astrocytes occurred in the ZFP group relative to the ZFPDelta group (**Fig. 5G,H)**. We compared this reduction in mHTT expressing S100β positive astrocytes at 11 weeks to their disease-dependent increase between 4-12 weeks (**Fig. 5G**). There were no mHTT expressing astrocytes in the control mice at any age, but in R6/2 mice, their proportion increased between 4 and 12 weeks (**Fig. 5G**). A linear fit to the data suggested that ZFPs reduced mHTT expression at 11 weeks to that expected for mice about

5 weeks old ($y = 0.42x - 1.85$, $R^2 = 0.95$; **Fig. 5G**). Since it takes ~1 week for the ZFPs to express, this suggests that ZFPs arrest the accumulation of mHTT within astrocytes almost as soon as the ZFP is expressed, resulting in reduced mHTT load at 11 weeks (**Fig. 5G**). We also quantified the number of medium spiny neurons (MSNs) containing mHTT by using the MSN marker DARPP32. We observed a modest reduction in the number of MSNs containing mHTT inclusions in mice injected with ZFP (**Fig. 5I,J**). This is an unexpected result and may suggest that handling of mHTT within neurons is non cell-autonomous and influenced by astrocytes. Alternatively, the ZFPs may be expressed at some low amount in MSNs. We also found a reduction in the size of the mHTT puncta as well as in their intensity (**Fig. 6A-E**) within astrocytes, and there was a reduction in the density of mHTT puncta in the bulk tissue (**Fig. 6F,G**). These results show that astrocyte specific ZFP-mediated mHTT lowering is effective and reduced the load of mHTT and the number of mHTT expressing astrocytes.

Astrocyte selective mHTT lowering with ZFPs reduced astrocyte molecular signatures

We performed astrocyte RNA-seq from NCAR and R6/2 mice injected with ZFP or ZFPDelta AAVs (**Fig. 7A**). AAVs were microinjected at 4 weeks and astrocyte specific RNA was purified at 11 weeks for sequencing (**Fig. 7A**). With a FDR < 0.05, for the ZFPDelta group, comparison of R6/2 vs NCAR resulted in 4878 DEGs in striatal astrocytes, whereas there were 4403 DEGs between R6/2 and NCAR in the ZFP group. By applying a FPKM cutoff of 10, the number of DEGs decreased to 1293 and 1175 in the ZFPDelta and ZFP groups, respectively (**Fig. 7B**). From these, 848 genes were differentially expressed, but shared between the groups, 444 were unique to the ZFPDelta group, and 326 were unique to the ZFP group (**Fig. 7C**).

We explored if the 62-core genes conserved across mouse models and human data (**Fig. 4D**) were rescued by ZFPs (relative to ZFPDelta). Sixty-one of the 62-core genes were found

within the shared genes between ZFP and ZFPDelta. We analyzed these DEGs by assessing the log_2 ratio for each gene (relative to NCAR) and plotting them as a fraction – as the log_2 ratio of the ZFP group divided by the log_2 ratio of the ZFPDelta group (**Fig. 7D**). If this ratio was 1, then this indicates that the ZFP had no effect on differential expression of that gene in R6/2 mice relative to ZFPDelta. If the ratio was > 1, this indicates that differential expression increased in the ZFP group relative to ZFPDelta. If, however, the ratio was < 1, this indicates that the differential expression observed between R6/2 and NCAR mice was reduced by the ZFP relative to the ZFPDelta group. Sixty-one of the 62-core genes displayed a fraction < 1 with a mean of 0.67 ± 0.01 and a median of 0.65 (**Fig. 7D, E**; $P < 0.0001$). In contrast, 61 randomly selected genes showed a ratio of 1.3 ± 0.3 with a median of 0.94, which was not significantly different from one (**Fig. 7E**; $P = 0.7088$). Overall, the data show that the magnitude of core astrocyte molecular signatures observed in HD mice was reduced by ZFP-mediated mHTT lowering. In accord, the magnitude of differential expression for many of the top 50-astrocyte DEGs in 3 month R6/2 mice was also diminished by mHTT lowering (fig. S14A-D).

Next, we evaluated the genes that were unique to the ZFPDelta group, because these may represent those associated with HD and completely rescued in the ZFP group (**Fig. 7C**). The top 25 are shown in fig. S14E and the top 20 pathways are shown in **Fig. 7F**. Within these pathways were mechanisms previously reported for HD pathology, such as protein ubiquitination, GABA and cAMP signaling. In contrast, fig. S14F shows the top 25 genes that were unique to the ZFP group relative to the ZFPDelta group, because these may represent those modulated during the process of mHTT lowering, in other words during reduction of molecular pathology. The top 20 pathways associated with these genes are shown in **Fig. 7G**, and include telomerase signaling, and pathways involved in reactive oxygen species and cell proliferation. We next evaluated the pathways that were shared between the unique genes in the ZFP and ZFPDelta groups with a view

to identifying reciprocal changes. Thirty pathways were identified as shared (**Fig. 7H**) and of these, three changed reciprocally in ZFP (towards activation) and ZFPDelta (towards inhibition) groups, as indicated by their opposite IPA z-score values. These were "Huntington's disease signaling", "ATM signaling" involved with DNA repair as well as "Wnt/β-catenin signaling". Furthermore, the top five upstream regulators of the 62-core genes using IPA were *Htt*, *Adora2a*, *Mapt*, *Hdac4* and *App*. *Htt* has obvious face validity. *Adora2a* encodes for the adenosine 2a GPCR (A2a) that is highly expressed in the striatum. In a cell-specific transcriptomic database, *Adora2a* is found in astrocytes and neurons (*28*), is detected in glia by electron microscopy and IHC (*29, 30*) and its deletion mainly from astrocytes leads to psychomotor and cognitive impairment (*31*). From RNA-seq, we found that *Adora2a* was downregulated in Q175 and R6/2 mice relative to controls (**Fig. 7J,K**), a finding confirmed by RNAscope (**Fig. 7L,M**). Moreover, ZFP expression reduced *Adora2a* differential expression relative to ZFPDelta (**Fig. 7N**). In parallel with mHTT lowering strategies, molecules that target *Adora2a*, *Mapt*, *Hdac4* and *App* within astrocytes may be useful as therapeutic targets to reduce astrocyte molecular dysfunctions in HD.

Discussion

Caused by a single known genetic defect that is found in astrocytes as well as neurons (*3*), HD represents an exemplar neurological disorder to explore basic astrocyte biology and its contributions to disease and striatal circuit function (*32*). Furthermore, there is broad interest in exploiting astrocytes to modulate the mechanisms that drive neurodegeneration (*2, 33-35*). In this study, we explored astrocytes agnostically at multiple stages in two mouse models of HD and in relation to data from HD postmortem samples. Our aim was to determine astrocyte molecular signatures in HD, and thoroughly analyze the data to identify the altered pathways. In summary, we found a core set of gene expression changes conserved across different HD models, at the level

of RNA and protein, in mouse and human. The gene ontology (GO) analysis of the conserved genes confirms that the functions altered in astrocytes comprise Ca^{2+} signaling, GPCRs, and neurotransmitter regulation (*6*), which predict regulation of grooming and locomotor behaviors with relevance to HD phenotypes in mouse models (*6, 36*). Although these gene expression changes and their predicted functions do not fall into a convenient mnemonic such as neurotoxic, our data nonetheless represent an unbiased differential gene and protein analyses that begins to define astrocyte dysfunction in HD.

The study contains some intrinsic limitations worth mentioning. First, although our data represent unbiased DEG analyses that begins to define astrocyte dysfunction in HD, it will be necessary to determine if correcting astrocyte dysfunction produces desirable effects in vivo in relation to behavior and disease phenotypes. Second, we have restricted our analyses to the striatum. However, mHTT is expressed throughout the body and it will be necessary to explore astrocytes in other brain areas with different degrees of pathophysiology to determine why the striatum is especially vulnerable to atrophy and degeneration. Third, we have explored the consequences of reducing astrocyte mHTT using ZFPs, but these tools need to be used to explore mHTT reduction in both astrocytes and neurons in order to systematically determine cell-specific contributions to disease. Fourth, in future studies it will be necessary to perform additional evaluations with fresh or carefully preserved postmortem human HD tissue in order to evaluate human pathophysiology. Fifth, and perhaps of most interest, our studies identify early astrocyte molecular signatures unrelated to reactivity, but it will be necessary to explore these pathways in humans. Viewed from these perspectives, our studies provide the impetus, database resources and rational for additional work to comprehensively explore astrocytes in HD. The findings also have broader implications for astrocyte contributions to CNS diseases, trauma and injury.

Astrocytes from mouse models (*6, 26*) and those derived from induced pluripotent stem cells from humans with HD (*37*) are known to show changes in potassium channels, neurotransmitter transporters, calcium signaling, morphology, metabolism, reactivity and spatial interactions with neurons (see Introduction). However, in most of these cases, the underlying mechanisms remain unclear and it is not known if these changes are correlative or causative with regards to HD-related pathophysiology. Furthermore, it remains to be established if these and other alterations are cell autonomous or secondary to changes in neurons. Unbiased systems biology approaches are necessary to shed light on these issues with carefully curated data from mouse models and humans. In relation to this, our data show that astrocytes in HD lose essential functions at early stages. These changes were progressive as the disease phenotypes developed and in some cases model-dependent. However, across the board, we found no evidence for strong astrocyte reactivity, especially at early stages, and little evidence supporting the notion that A1 neurotoxic astrocytes cause neuronal death in HD. In accord with these interpretations, in humans around half of striatal neurons are known to be lost at grade 1 (*17*), and yet we found no evidence for A1 reactivity at this grade. At later stages, there was evidence for increased reactivity associated gene expression, but these were not A1 specific and perhaps more consistent with neuroinflammation (*38*). Interestingly, a well-defined neuroinflammatory insult (LPS) also provided little evidence for uniquely A1 neurotoxic astrocytes in the striatum, which suggests that the A1 and A2 classification that has been proposed might not apply to all brain areas even in the absence of brain disease. These are important considerations for future work and emphasize the brain area-specific nature of astrocytes: perhaps evaluations that derive conclusions without considering regional variation are likely to be problematic to interpret in relation to disease-specific mechanisms. The reactivity-related changes in astrocytes that do occur are only present in the late stages and are unlikely to contribute to early HD-related pathogenesis or drive neuronal loss. It remains to be

determined if early astrocyte changes can drive synaptic loss in HD mouse models and in humans with HD. Nonetheless, the early stage astrocyte changes may contribute to psychiatric and cognitive aspects of HD. If so, targeting these pathways may provide opportunities to develop therapies for symptoms associated with early stage HD, as has been suggested in recent studies with HD model mice (*21*).

The core astrocyte molecular signature of 62 altered genes we identified across mouse and human data was ameliorated by astrocyte-specific mHTT lowering using ZFPs, revealing astrocyte-specific changes during the progression and reversal of molecular pathology. These data suggest that some astrocyte dysfunctions in HD are likely to be cell-autonomous (*27*), but additional work is needed to rigorously explore this possibility. In view of our findings, we suggest that neuronal death is likely caused by widespread mHTT expression within neurons and exacerbated by the accompanying loss of astrocyte essential functions that initially become dysfunctional and then increasingly reactive as the disease progresses to striatal atrophy. Such astrocyte dysfunctions could include ion homeostasis ([MM1] K^+ or Ca^{2+}), neurotransmitter transport such as for glutamate and GABA, metabolism, cell morphology and/or GPCR signalling. Interestingly, analyses of potential upstream regulators of the 62-core molecular changes revealed *Adora2a* and *Hdac4*, both of which are therapeutic targets in HD (*39-41*). These findings suggest that early and progressive molecular pathways we identified within astrocytes that led to dysfunctions could be exploited to delay disease progression. We do not diminish the importance of cell autonomous neuronal damage, and suggest that the most fruitful approaches may include astrocytic interventions in parallel with neuronal rescue and repair strategies or with pan-cellular mHTT lowering approaches using ZFPs that target most or all brain cells. Translationally, a combination of strategies aimed at correcting dysfunctions of neurons, astrocytes and other glia are likely to be more effective than targeting any single cell type alone.

Our findings and database resources provide a rich source of information to formulate and test specific hypotheses in relation to therapeutic targets for HD and for additional mechanistic studies in other disorders. We propose that astrocytes change in a measurably disease, progression and brain region-specific manner (*18*), and in ways that could be exploited to reveal new therapeutic targets in diverse brain diseases and to reveal context specific mechanisms. Restoring brain tissue homeostasis by targeting astrocytes may prove useful in a variety of neurodegenerative diseases, including HD.

Materials and Methods

Study design

We performed striatal astrocyte specific RNA-seq from two HD mouse models. The first is a transgenic model (R6/2) considered to be severe and that likely mimics juvenile onset HD (*10*). The second is a knock-in model (Q175), which has a milder phenotype and better reflects adult onset HD (*19*). We assessed each mouse model at three different stages of the disease in relation to cognate controls, referred to as wild type (WT) for Q175 and non-carriers (NCAR) for R6/2 mice. We sequenced astrocyte specific RNA, but also that from the soup of all cells akin to bulk tissue (*18, 21*). We also assessed astrocyte gene and protein expression in human HD samples and in the allelic series of HD mouse models (www.HDinHD.org). Sample sizes were based on similar previously published work. No data, including outliers, were excluded from the analyses. RNA-seq was blinded for the person preparing the cDNA library and sequencing run. Detailed methods are provided in the Supplementary Material.

Statistical analyses

Raw replicate values for all experiments and statistical test results are provided in Data S5. The results of statistical comparisons, n numbers and *P* values are shown in the figure panels or figure legends with the average data. When the average data are reported in the text, the statistics are reported there. Statistical tests were run in GraphPad Instat 3. Summary data are presented as mean ± s.e.m. along with the individual data points. Note that in some of the graphs the bars representing the s.e.m. are smaller than the symbols used to represent the mean. For each set of data to be compared we determined within GraphPad Instat whether the data were normally distributed or not. If they were normally distributed, we used parametric tests. If the data were not normally distributed, we used non-parametric tests. Paired and unpaired Student's two-tailed *t* tests (as appropriate) and two-tailed Mann–Whitney tests were used for most statistical analyses with significance declared at $P < 0.05$, but stated in each case with a precise *P* value. When a *P* value is reported in the figures, then the test used is reported in the figure legend. When the *P* value was less than 0.0001, it is stated as such. *N* is defined as the numbers of cells, sections or mice throughout on a case-by-case basis; the unit of analysis is stated in each figure panel, in the text or figure legend. A statistical FDR value < 0.05 was used for all RNA-seq analyses. No data points were excluded from any experiment.

Supplementary Materials

Materials and Methods

Fig. S1: Data to support assessments of astrocyte reactivity in human and mouse HD shown in Fig. 1 of the main text.

Fig. S2: Rpl22-HA expression in d.l. striatum.

Fig. S3: Overall numbers of differentially expressed astrocyte genes for Q175 and R6/2 mouse models versus their cognate controls.

Fig S4. Astrocyte differential gene expression in Q175 vs WT and R6/2 vs NCAR at the three stages of study.

Fig. S5: Overall numbers of differentially expressed astrocyte genes in HD versus control with the application of different threshold criteria to analyze the data.

Fig. S6: Assessing astrocyte reactivity in HD mouse models with RNA-seq.

Fig. S7: Summary data showing how chosen pathways of interest in astrocyte biology change in mouse models of HD.

Fig. S8: Pathways altered within astrocytes in HD model mice.

Fig. S9: Assessments of early gene expression changes in HD.

Fig. S10: Weighted gene co expression network analysis (WGCNA) to identify progressive alterations in astrocytes from HD mouse models.

Fig. S11: WGCNA modules with gene expression profiles that go in opposite directions in controls when compared to HD mice.

Fig. S12: Common DEGs between Q175 vs WT and R6/2 vs NCAR.

Fig. S13: Differential expression of the 62-core genes identified in the main manuscript in the allelic series proteomics datasets.

Fig. S14: Altered relative expression of the top 50 DEGs from 3 m R6/2 mice in ZFPDelta and ZFP groups.

Data S1: This Excel file includes the DEGs with FDR < 0.05 and FPKM > 10 for Q175 vs WT at 2, 6 and 12 months of age. Upregulated genes are highlighted in pink, downregulated in blue.

Data S2: This Excel file includes the DEGs with FDR < 0.05 and FPKM > 10 for R6/2 vs NCAR at 1, 2 and 3 months of age. Upregulated genes are highlighted in pink, downregulated in blue.

Data S3: This Excel file lists the genes that belong to each of the WGCNA modules identified in fig. S10.

Data S4: "ZFP DEGs" - this table includes the shared and unique DEGs between ZFPDelta and ZFP (R6/2 vs NCAR, FDR < 0.05, FPKM > 10). "IPA pathways" - this table includes the IPA pathways (P < 0.05) for the ZFPDelta - unique DEGs and ZFP - unique DEGs (R6/2 vs NCAR, FDR < 0.05, FPKM > 10). "The 62-core DEG upstream regulators" - this table contains the 62-core DEG upstream regulators identified with IPA.

Data S5: This excel file (consisting of three tables) contains all the raw replicate values that were used to generate the graphs shown in the figures. It also contains all the statistical tests and results

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Acknowledgments: Thanks to Michael Levine and Poonam Sachan for sharing mice. Thanks to the Khakh laboratory and Drs. Cachope, Beaumont and Munoz-Sanjuan (CHDI) for feedback.

Thanks to Fuying Gao for assistance with RNA-seq data processing, and the UCLA Neuroscience Genomics Core for sequencing. Thanks to the HDinHD support team for their assistance. **Funding:** This work was supported by CHDI, the National Institutes of Health (NS111583 and MH104069 to BSK) and the American Heart Association (16POST27260256 to XY). We acknowledge the support of the NINDS Informatics Center for Neurogenetics and Neurogenomics (P30 NS062691 to GC). The project described was supported in part by the Genetics, Genomics and Informatics Core of the Semel Institute of Neuroscience at UCLA, which is supported by IDDRC grant number U54HD087101-01 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development. **Author contributions:** B D-C performed experiments in Figs 1-4. MRG performed experiments in Figs 5-7. XY performed reference experiments with LPS. B D-C performed RNA-seq data analyses and trained MRG in these approaches. GC helped analyze the RNA-seq and gene expression data. BSK conceived the study, directed the experiments and designed the layout of the figures with help from BD-C and MRG. BSK wrote the paper with help from B D-C and MRG. All authors contributed to the final version. **Competing interests:** the authors have nothing to declare. **Data and materials availability:** Raw and normalized RNA-seq data from both mouse models at 3 ages (input and IP) have been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE124846. FPKM RNA-seq values are also provided in Excel files as Data files S1-4. The human and allelic series data are available (https://www.hdinhd.org/). Our data will be shared via our own website http://astrocyternaseq.org. Other data not covered by these resources are present in the main text or in the supplementary material.

FIGURE LEGENDS

Fig. 1. Astrocyte reactivity in HD mouse models and in human samples. (**A**) Human RNA datasets analyzed. (**B**) Mouse RNA and protein datasets analyzed. (**C, D**) Human caudate nucleus differential expression (DE) (*14*) of 35 astrocyte reactivity genes **(C)** and genes known to be overexpressed in activated microglia (*16*), including A1 type inducing genes (*12*) **(D)**. DE is shown in $\log_2$ ratio color scale (red for upregulated, blue for downregulated) for each disease grade ranging from 0 to 3. * indicates genes that are significantly different (FDR < 0.05) between HD and control. The boxed genes (green) have a $\log_2$ ratio > 2. On the right of **(C)**, a bar graph shows the percentage of the top 100-striatal astrocyte enriched genes (enriched in IP vs input) that are differentially expressed at each HD grade. (**E**) RNA qPCR DE of the top astrocyte reactivity genes in the dorsolateral (d.l.) striatum of mouse models of HD (Q175 and R6/2) at three different disease stages. LPS injected mice were used as a positive control. Gene expression was normalized to *Gapdh* (left) or *Rplp0* (right) and expressed as color-coded –ddCt (red upregulated, blue downregulated). * indicate the genes that are significantly different ($P < 0.05$). N = 3-7 mice per condition. (**F, G**) Striatum allelic series RNA-seq DE of the top astrocyte reactivity genes **(F)** and genes that are known to be overexpressed in active microglia (*16*), including A1 inducing genes (*12*) **(G)**, at three different ages, 2 months (m), 6 m and 10 m shown in $\log_2$ ratio color scale (red for upregulated and blue for downregulated). Genes not found in the datasets are colored in grey. * indicate genes that are significantly different (FDR < 0.05). (**H, K**) GFAP IHC of 12 m WT and Q175 **(H)** or 3 m NCAR and R6/2 **(K)** d.l. striatum. (**I, L**) GFAP IHC intensity x area quantification for WT and Q175 at 2 m, 6 m, 12 m **(I)** or R6/2 at 1 m, 2 m, 3 m **(L)**. (**J, M**) GFAP western blot protein quantification, normalized to βIIITubulin, and example gel (below) for WT and Q175 at 2 m, 6 m, 12 m **(J)** or NCAR and R6/2 at 1 m, 2 m, 3 m. Average data are shown as mean ± SEM. For panels **E, I, J, L** and **M** and unpaired Student's *t* test was performed if the values

where normally distributed and a Mann-Whitney test was used if they were not (*P* values and n numbers are on the figure panels and in Data S5).

Fig. 2. Astrocyte specific AAV2/5 RiboTag. (**A**) Astrocyte specific RNA-seq from HD mice. (**B, C**) IHC images of 6 m WT d.l. striatum as control for Q175 (**B**), and 2 m NCAR d.l. striatum as control for R6/2 mice (**C**). **D, E.** % of S100β$^+$ or NeuN$^+$ cells that colocalized with Rpl22-HA for WT, Q175 (**D**), NCAR and R6/2 mice (**E**), (n = 3 mice). (**F, G**) Gene expression (in FPKM) of brain cell-type markers in WT (**F**) and NCAR (**G**). (**H, I**) Relative RNA-seq expression (z-score) of the top 200 adult-striatum astrocyte-enriched genes (enriched in IP vs input with FPKM > 10 (*18*)) **(H)** or whole-striatum enriched genes (depleted in IP vs input) **(I)** in WT and NCAR samples. (**J, K**) PCA of WT and Q175, 2, 6 and 12 m **(J)** or NCAR and R6/2, 1, 2 and 3 m **(K)**. RNA-seq was performed in 4 replicate mice. Open circles are raw data with closed circles indicating mean ± s.e.m.

Fig. 3. Top astrocyte DEGs in Q175 and R6/2 HD mice. (**A**) Chord diagram of common genes across datasets. The thickness of the chords is proportional to the number of genes. (**B, C**) Overlap between the top 50 up and downregulated DEGs in Q175 (FPKM > 10) at 2 m, 6 m and 12 m **(B)** and in R6/2 at 1 m, 2 m and 3 m **(C)**. (**D, E**) Heat maps of $\log_2$ ratio of the top 50 down (**D**) and upregulated (**E**) DEGs in Q175. (**F, G**) Heat maps of $\log_2$ ratio of the top 50 down (**F**) and upregulated (**G**) DEGs in R6/2. DEGs within the top 50 most different are marked with a *. DEGs that are not within the top 50 are marked with #. Genes that are consistently differentially expressed in human samples (*14*) are highlighted in green font. Astrocyte enriched genes (IP vs input with FPKM > 10 (*18*)) are pointed with black ($\log_2$ ratio > 1) or grey arrows $\log_2$ ratio > 0 < 1. (**H**) Main functions performed by the top 50 down or upregulated DEG (FPKM >10). Color intensity represents the proportion of down or upregulated genes that participate on the function shown on

the left. The aggregate score (A. S.) on the right of each heat map is the sum of the % of down or upregulated DEG in each dataset for each function.

Fig. 4. Molecular signatures of astrocytes in HD. (**A**) Number of differentially expressed proteins from mouse whole striatum proteomic data (HDinHD; www.hdinhd.org): 2 m, 6 m and 10 m for Q175 vs WT, and 1 m, 2 m and 3 m for R6/2 vs NCAR. (**B-D**) Overlap of the astrocyte DEGs, in Q175 **(B)**, R6/2 **(C)** and common within datasets (**D**), with the differentially expressed proteins in **A**. (**E-G**) Overlap of the number of astrocyte DEGs in Q175 **(E)**, R6/2 **(F)** and common within datasets (**G**), with DEGs in human caudate. Overlaps are shown in light blue for down and light pink for upregulated genes. (**H**) Number of genes common between the four symptomatic astrocyte RNA datasets (6 m, 12 m Q175, 2 m, 3 m R6/2), mouse proteomics and human RNA. (**I**) DE (log_2 ratio) heat map of the 62 genes common across all datasets (intersection in **H**). (**J**) 62 gene DE in striatum allelic series RNA-seq data. Heat maps show the DE in log_2 ratio color scale (red for upregulated and blue for downregulated). Non-DEG (FDR > 0.05) are colored in grey. The two astrocyte enriched genes are pointed out with black arrows (log_2 ratio > 1, in comparisons of striatal IP vs input with FPKM > 1). The other DEGs were expressed in astrocytes, but not enriched.

Fig. 5. Astrocyte mHTT lowering with astrocytic expression of ZFP transcriptional repressors. (**A**) Cartoon of AAVs used: one AAV expressed a ZFP m*Htt* transcriptional repressor. The other was identical, but was non-binding and served as control. The ZFPs were HA-tagged and tdTomato was also expressed. (**B, C**) Colocalization between S100β **(B)**, or NeuN **(C)**, and HA-tagged ZFP. The lower scatter plot summarizes quantification of colocalization for both ZFP and ZFPDelta. (**D**) Timeline for ZFP AAV injection and IHC assessment. (**E, F**) mHTT

immunostaining in S100β positive astrocytes **(E)**, or DARPP32 positive MSNs **(F)**, from ZFPDelta and ZFP groups. In each image, the inset shows a zoomed in area from the picture. (**G**) The left hand scatter plot shows that ZFP reduced the mHTT load in S100β positive astrocytes relative to the ZFPDelta group. The scatter plot to the right shows stage-dependent increases in mHTT load within S100β positive astrocytes. (**H**) The scatter graph shows that mHTT load reduction was extremely reproducible across five separate batches of mice. (**I**) Plot shows that ZFP subtly reduced the mHTT load in DARPP32 positive MSNs relative to the ZFPDelta group. (**J**) The scatter graph shows that mHTT load reduction with MSNs was variable across five separate batches of mice. The open circles are raw data with closed circles indicating mean ± s.e.m. For panel G, an unpaired Student's *t* test with Welch correction was performed. For panel I, an unpaired Student's *t* test was performed (*P* values and n numbers are on the figure panels and in Data S5).

Fig. 6. Analysis of mHTT puncta following mHTT lowering in astrocytes. (**A**) mHTT puncta in tdTomato expressing astrocytes from ZFP and ZFPDelta groups. (**B, C**) Examples of 10-astrocyte mHTT puncta from ZFP and ZFPDelta groups. (**D, E**) Graph plots astrocyte mHTT puncta size **(D)** or puncta intensity **(E)** in ZFP and ZFPDelta groups. (**F**) Representative images of mHTT puncta in striatal tissue in ZFP and ZFPDelta groups. (**G**) Graph of mHTT puncta density within the tissue sections for ZFP and ZFPDelta groups. In this figure, the open circles are raw data with closed circles indicating mean ± s.e.m. For panels **D** & **E**, a Mann-Whitney test was performed. For panel **G**, an unpaired Student's *t* test with Welch correction was performed (*P* values and n numbers are on the figure panels and in Data S5).

Fig. 7. HD-associated astrocyte molecular signatures following mHTT lowering.

(**A**) Cartoon of the approach. (**B**) Number of DEGs in the IP fraction (FDR < 0.05, FPKM > 10) from R6/2 and NCAR in ZFPDelta-injected control group and ZFP-injected treatment group. (**C**) Comparison of the DEGs between ZFPDelta (1293 DEGs) and ZFP (1175 DEGs) groups. (**D**) On the left, heat map showing the DE in log_2 ratio of the 62-core DEGs, shown in Fig. 4, in the ZFPDelta and ZFP samples. The bar graph on the right presents the ratio, i.e. the ZFP log_2 ratio divided by the ZFPDelta log_2 ratio for each gene. (**E**) Plot showing the ratios of ZFP vs ZFPDelta for 61 of the 62-core DEGs and another 61 randomly selected genes from our dataset. The ratios where compared to 1, as theoretical value for no difference between ZFP and ZFPDelta groups. (**F**) Top 20, of the 108 IPA pathways ($P < 0.05$) for the 444 ZFPDelta–unique DEGs. (**G**) Top 20, of 120, IPA pathways ($P < 0.05$) for the 326 ZFP–unique DEGs. (**H**) The 30 IPA pathways that were identified in both ZFPDelta–unique and ZFP–unique IPA analyses. The heat maps in F-H show the IPA z-score which indicates if the pathway is predicted to be inhibited (blue) or activated (red). Three pathways, highlighted in green, were predicted to be inhibited in ZFPDelta, but activated in ZFP expressing astrocytes. (**I**) Top 5 upstream regulators for the 62-core DEGs. (**J & K**) *Adora2a* RNA expression (FPKM) in IP samples for all Q175 vs WT (**J**) and R6/2 vs NCAR (**K**) samples. * indicates the ages at which *Adora2a* was found to be differentially expressed (FDR < 0.05). (**L**) Representative images of *Adora2a* RNA-scope (magenta) and astrocyte GCaMP6f expression IHC (green) in 3 m NCAR and R6/2 astrocytes. (**M**) Quantification of the coverage of *Adora2a* signal in area % of 3 m NCAR or R6/2 astrocyte cell bodies. (**N**) Ratio ZFP/ZFPDelta for *Adora2a*, which was lower than 1. For panel E, a Wilcoxon signed rank test was performed. For panel **M**, a Mann-Whitney test was performed (*P* values and n numbers are on the figure panels and Data S5).