

Identification of neurodevelopmental organization of the cell populations of juvenile Huntington's disease using dorso-ventral HD organoids and HD mouse embryos

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This manuscript describes the generation of a fused dorsal-ventral organoid system to model interactions between the cortex and striatum to study the onset and progression of Huntington's disease (HD) and other neurodegenerative disorders. While this approach is **valuable**, further methodological and analytical work is needed to fully support the interpretations and claims of the authors. **Incomplete** evidence suggests choroid plexus (ChP) abnormalities form a significant component of HD pathogenesis.

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Abstract

Huntington's disease (HD) is a polyglutamine neurodegenerative disease involving pathogenesis within the striatum and cerebral cortex and a neurodevelopmental component, particularly in juvenile HD form (JOHD). We established a fused HD dorsal-ventral system, imitating the cortex and striatum interaction in a single organoid to discover neurodevelopmental impairments at the level of cell populations. We found a range of early pathogenic phenotypes indicating that brain development in HD is affected by impaired neurogenesis. The phenotypes occurred already in early-stage 60-day organoids and the brain of humanized mouse embryos, at time of the beginning of the neurogenesis and choroid plexus development. We demonstrated that HD organoids and HD mouse embryonic brains had gene expression profiles of impaired maturation of neurons and increased expression of genes responsible for proliferation compared to genes responsible for differentiation in control organoids. By using scRNA-seq, the choroid plexus population was highly abundant in

HD organoids and embryonic brains. Cortical and choroid plexus cell populations in HD organoids and brains co-expressed genes responsible for *HTT* function (mitotic spindle and cilia). The impaired maturation and the increased occurrence of the choroid plexus populations were mitigated in our compensatory model, mosaic dorsal/ventral (D/V) or V/D HD/control organoids. Finally, we found that TTR protein, a choroid plexus marker, is elevated in the adult HD mouse serum, indicating that TTR may be a promising marker for detecting HD. In summary, the fused dorso-ventral HD organoids identify a spectrum of neurodevelopmental features, including increased proliferation and delayed cell maturation. We demonstrate that the choroid plexus population is characteristic of aberrant HD neurodevelopment, and contains TTR marker, which can be translated as a blood marker in HD.

1. Introduction

Huntington's disease (HD) is a fatal, dominantly inherited neurodegenerative disease that affects mainly the central nervous system, causing the loss of neurons in the striatum and cerebral cortex (Cudkowicz and Kowall, 1990 [↗](#); Reiner et al., 1988 [↗](#)). Symptoms may occur years or even decades prior to diagnosis, and the disease often worsens as the number of CAG repeats in the huntingtin gene increases (Quarrell et al., 2013 [↗](#)). In rare cases, particularly with more than 60 repeats, the disease can occur before the age of 20, known as juvenile HD (JHD) (Fusilli et al., 2018 [↗](#)), progresses more rapidly, and has different manifestations than adult-onset HD. Studies suggest that pre-manifest HD patients can develop cognitive, psychiatric, and motor impairments long before diagnosis (Paulsen et al., 2014 [↗](#); Quaid et al., 2017 [↗](#)). In addition, decades before adult-onset the gene deregulation involved in neurodevelopment and neuronal degeneration can also occur, in both, mouse models and patients (Hodges et al., 2006 [↗](#); Labadorf et al., 2015 [↗](#); Milnerwood et al., 2006 [↗](#); Schippling et al., 2009 [↗](#)). One of such phenotype that occurs years before overt HD symptoms is enlarged ventricular volume in the brain (Hobbs et al., 2010 [↗](#); Jahanshahi et al., 2022 [↗](#)). Also, juvenile HD demonstrates an accumulation of various brain defects such as obvious ventricular enlargement. The symptom is characteristic for neurodevelopmental diseases such as autism spectrum disorders (ASD) where it occurs together with abnormalities of the choroid plexus (ChP) and the formation of cysts of ependymal origin in early brain development (Movsas et al., 2013 [↗](#)). Similarly, ChP volume was significantly increased in psychosis patients (Lizano et al., 2019 [↗](#)). Alzheimer's disease (AD) is associated with numerous changes in ChP morphology and function, such as changed metabolic activity and reduced toxins clearance (Krzyzanowska and Carro, 2012 [↗](#); Marques et al., 2013 [↗](#); Spector and Johanson, 2013 [↗](#)). The ChP is an important physiological barrier and the principal source of cerebrospinal fluid (CSF) responsible for clearance of toxic products from central nervous system (CNS) (Lehtinen et al., 2013 [↗](#); Redzic et al., 2005 [↗](#)).

Potential neurodevelopmental conditions such as autism (Birey et al., 2017 [↗](#); Mariani et al., 2015 [↗](#)), microcephaly (Tiscornia et al., 2011 [↗](#)), HD (Conforti et al., 2018 [↗](#)) and BBB (Pellegrini et al., 2020 [↗](#)) were already investigated by using small three-dimensional (3D) brain organoids (Lancaster et al., 2013 [↗](#)). Recently, the brain organoid approach has been enriched with fused cerebral organoids which recreate the dorsal-ventral forebrain axis (Bagley et al., 2017 [↗](#)). In HD, loss of neostriatal volume and medium spiny neurons (MSN) goes along with pyramidal neuron loss in the neocortex (Vonsattel et al., 2011 [↗](#)). The combination indicates complex HD pathogenesis, including various cellular and communication impairments between the dorsal (cortex) and ventral (striatum) regions particularly during forebrain development (Rangel-Barajas and Rebec, 2016 [↗](#)). Therefore, assembloids of dorso-ventral forebrain origin are an ideal model for studying the interactions between the most important and HD-involved brain regions. Brain organoids originating from juvenile HD cells would be particularly useful for defining neurodevelopmental HD brain pathogenesis at several levels, such as the molecular mechanisms, cellular organization of brain and blood-brain barrier (BBB) and choroid plexus impairments.

In our work, we have established a dorso-ventral HD juvenile fused organoids system to define the complex neurodevelopmental impairments at the levels of molecular biomarkers, cellular differentiation and interactions, and structural changes in HD brain. We showed that assembling the dorso-ventral axis by fusing HD organoids differentiated towards the cerebral cortex (CTX) and striatum (STR) can uncover distinct early brain neurodevelopment in HD. We found changes in the choroid plexus (ChP) in HD brain organoids and embryos of mouse models at the level of composition of cell populations, the presence of ChP pseudo organs in striato-cerebral organoids, and the occurrence of the ChP biomarkers in HD fused organoids and blood serum of HD mice. We demonstrate that ChP abnormalities are an essential neurodevelopmental component of HD pathogenesis and that the markers of ChP in blood serum can be developed as markers of early HD.

2. Results

2.1. Juvenile HD dorso-ventral brain organoids demonstrate markers of early developing cortex and striatum

The generation of our control and HD dorso-ventral forebrain organoids from human juvenile HD iPSC lines is demonstrated on the scheme in [Fig. 1A](#). In brief, neurospheres were primed either by chemical inhibition of SHH by smoothened receptor inhibitor cyclopamine A for obtaining dorsal organoids and by WNT inhibition by IWP2 and priming SHH by agonist SAG to ventral organoids which were later fused and cultivated to model the early pseudo tissue of cerebral cortex (CTX) assembled with the striatum (STR). Since we pursued to model early brain development, we established day 60 as the maturing endpoint for our control and HD organoids. Importantly, we also generated mosaic organoids, which were our compensatory model to assess the contribution of dorsal and ventral parts in the development of HD phenotypes in fused organoids. [Fig. 1B](#) presents a representative slice of our HD fused brain organoid stained for TBR1, a marker of the dorsal forebrain and the scheme of the coronal embryonic brain section showing the dorsal-ventral gradients.

First, we used unfused dorsal or ventral 60-day-old brain organoids to confirm the successful proliferation and maturation of various forebrain regions. We assessed the expression of specific cortical and striatal markers (Supp. Fig. 1). The separately primed organoids predominantly differentiated either to dorsal (*TBR1* and *PAX6*) or ventral markers (*NKX2.1*, *DLX2* and *GSX2*) as demonstrated via qPCR method (Supp. Fig. 1A). Subsequently, to assess the contribution of the cerebral cortex and striatum parts in 60 day fused cortico-striatal assembloids we detected their early markers by immunostaining (Supp. Fig. 1B). We detected immunostaining for TBR1 and PAX6, which are markers of ventricular zone (VZ) and subventricular zone (SVZ) of the developing neocortex (respectively), as dorsal forebrain markers. For examining ventral organoid parts, we detected the immunofluorescence staining of DLX2 and NKX2.1, markers of medial ganglionic eminence (MGE), and GSX2, marker of lateral ganglionic eminence (LGE). We also detected relatively late markers of differentiated neurons such as NEFH and SMI-32, indicating expression of forebrain markers, as well as other neuronal markers (Supp. Fig. 1B). Altogether, these results confirm the successful generation of fused dorso-ventral forebrain organoids, which are suitable to study neurodevelopmental aspects of HD pathology.

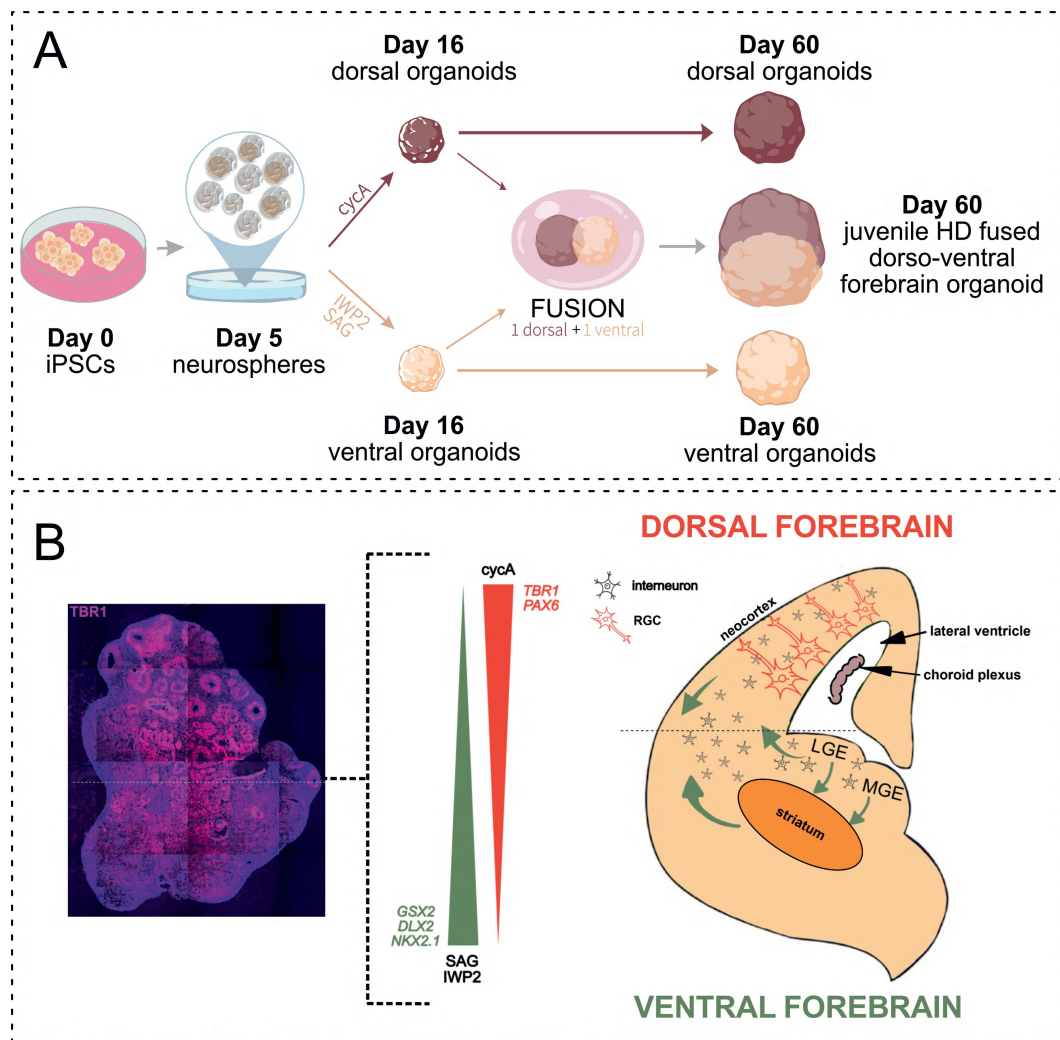


Figure 1.

Scheme of the culture system generation of fused dorso-ventral HD and control forebrain organoids.

A) The neurospheres were formed from iPSC control and HD juvenile lines and were primed either by SHH inhibition by smoothed inhibitor cyclopamine A for obtaining dorsal brain patterning and by chemical inhibition of WNT by IWP2 and activation of SHH by agonist SAG for obtaining ventral brain cell lineages. On DIV 16, the dorsal and ventral primed spheres were fused and cultivated to model the early pseudo tissue mimicking the cerebral cortex (CTX) assembled with the striatum (STR). To model early brain development, we established day 60 as the maturing endpoint for our control and HD organoids. The mosaic organoids from dorsal and ventral priming of HD and control lines were generated to produce a compensatory model to assess the contribution of dorsal and ventral parts in the development of HD phenotypes in fused organoids. B) A representative slice of HD fused brain organoid stained for TBR1 protein, a marker of the dorsal forebrain, and the scheme of the coronal embryonic brain section showing the dorsal-ventral gradients and their respective small molecules used.

2.2. Fused juvenile HD brain organoids are larger and contain prominent PAX6 neuronal stem cell rosettes, which are diminished in control and mosaic organoids

We examined the morphology and quantified the sizes of HD, mosaic, and control organoids ([Fig. 2](#)). The 109Q HD organoids were significantly larger and were remarkably more complex in their shape than all control fused organoids. 60-day-old HD organoids were significantly larger in size ($p\text{val.} < 3.35 \times 10^{-5}$) than control organoids ([Fig. 2A and B](#)). On average, mosaic 21D/71V organoids were close in size to control organoids, while the opposite configuration of mosaic 71D/21V was close in size to HD organoids ([Fig. 2B](#)). All post-hoc test p values are presented in [Fig. 2](#).

In the developing brain, there is an expression of the *PAX6* marker, which indicates the presence of a population of early progenitors, and the expression of the *TBR1* marker, which indicates the presence of a population of early-born neurons. Our qPCR (Supplement 1A) and RNA-seq DEG results detected the *TBR1* [$p\text{val} = 0.012387$; $\log(\text{FC}) = \sim 2.16$] and *PAX6* [$p\text{val} = 0.01252$; $\log(\text{FC}) = \sim 1.34$] markers upregulations in 60 days fused brain HD organoids. To confirm the presence of the *PAX6*-positive and *TBR1*-positive populations in our organoids, we performed an immunostaining protocol using the same type of organoids as used for scRNA-seq. Surprisingly, the immunostaining results on 60 days fused organoids ([Fig. 3](#)) demonstrate the occurrence of the large number of *PAX6*-positive neural rosettes lying close to each other and the occurrence of the separate layers of *TBR1* expression in HD fused organoids (71D/71V & 77D/77V). In contrast, the control fused organoids (17D/17V & 21D/21V) demonstrated only several scattered rosettes on organoid slices. Our results demonstrate that the development of the HD organoids is directed towards a more proliferative character of early neural development with the occurrence of a vast number of progenitors in rosettes.

The control organoids present lower expression of *PAX6* and *TBR1*, indicating a more balanced development of structures and their earlier neuron differentiation and maturation. Importantly, in the mosaic organoids, we observed the regression in the HD phenotype represented by reduced neural rosette occurrence, which is consistent with the results from GSEA (Supp. Fig. 2 A; Supp. 1).

2.3. Prominent deregulations of cell population markers of choroid plexus, inhibitory neurons, and glial cells in juvenile HD organoids

We analyzed the publicly available scRNA-seq data *in silico* to select markers of the most common brain population of neuronal and non-neuronal cells for further analysis of their expression levels in dorso-ventral HD and control organoids ([Fig. 4](#)). Another step in the inclusion of markers for analysis was the manual selection, population markers likely affected in the developing HD brain. *TTR*, *H3ST4*, *PPP1R1B*, *GPR88*, *NOS1*, *NXPH1*, *CKB*, *APOE* and *GRIN3A* genes were selected. Additionally, using an *in silico* bioinformatic decision tree and *HTT* expression level approach (described in Material and Methods), *ATOX1*, *FOXP1*, *LIMK2*, and *SMARCA4* marker genes of cell populations were selected.

Selected genes were general markers associated with all basic types of brain cells, including inhibitory and excitatory neurons, astrocytes and oligodendrocytes, tanycytes, choroid plexus, nitrergic neurons, and neuronal progenitors. Firstly, we compared the expression of several inhibitory neuron markers and showed the deregulated expression of five markers. *LIMK2* showed upregulation in all HD organoids compared to 21Q control organoids (ANOVA $p\text{val} = \sim 0.0007$). *NXPH1* showed highly significant downregulation in all HD lines compared to 21Q and 33Q control organoids (ANOVA $p\text{val} = \sim 3.31 \times 10^{-6}$). Other markers of early inhibitory neurons, such as *FOXP1*, *GRIN3A*, and *CKB*, have demonstrated inconsistent or non-significant outcomes. Next, we focused on analyzing the expression of selected choroid plexus (ChP) and tanycyte

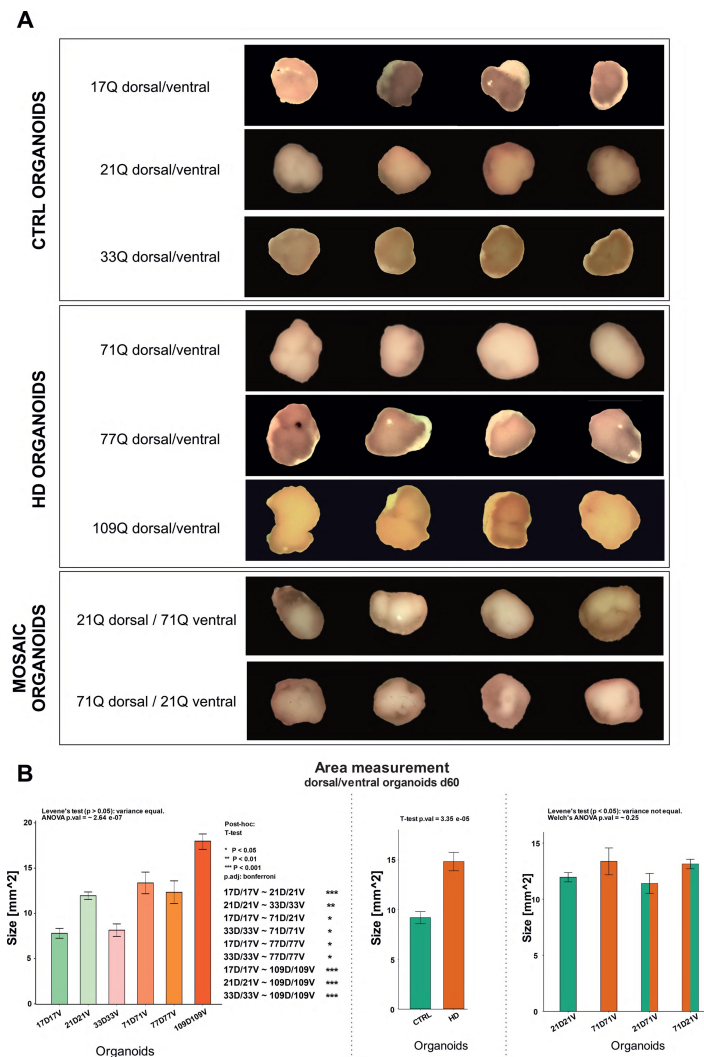


Figure 2.

Macroscopic organization of 60-day-old HD fused organoids shows their less regular structure and increased sizes.

A) Macroscopic 2D images of the fused dorsal/ventral control, HD organoids, and mosaic HD/control organoids from the iPSC lines used to generate the organoids; B) Area measurement graphs demonstrated data collected from 2D images of the organoids. The sizes of dorsal/ventral fused HD vs. control organoids were significantly greater and is also depicted as C) aggregated data panel where all control organoid sizes were plotted against HD organoid sizes (Levene's test $p > 0.05$: variance examination: equal; ANOVA $p.val = \sim 0.0086$; posthoc: T-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). The sizes of the mosaic organoids vs control and HD organoids did not reach statistical significance; however, on average, 21D/71V was close in size to control organoids, and the opposite configuration of mosaic 71D/21V was close in size to HD organoids. The area [mm²] of each organoid was measured in ImageJ software.

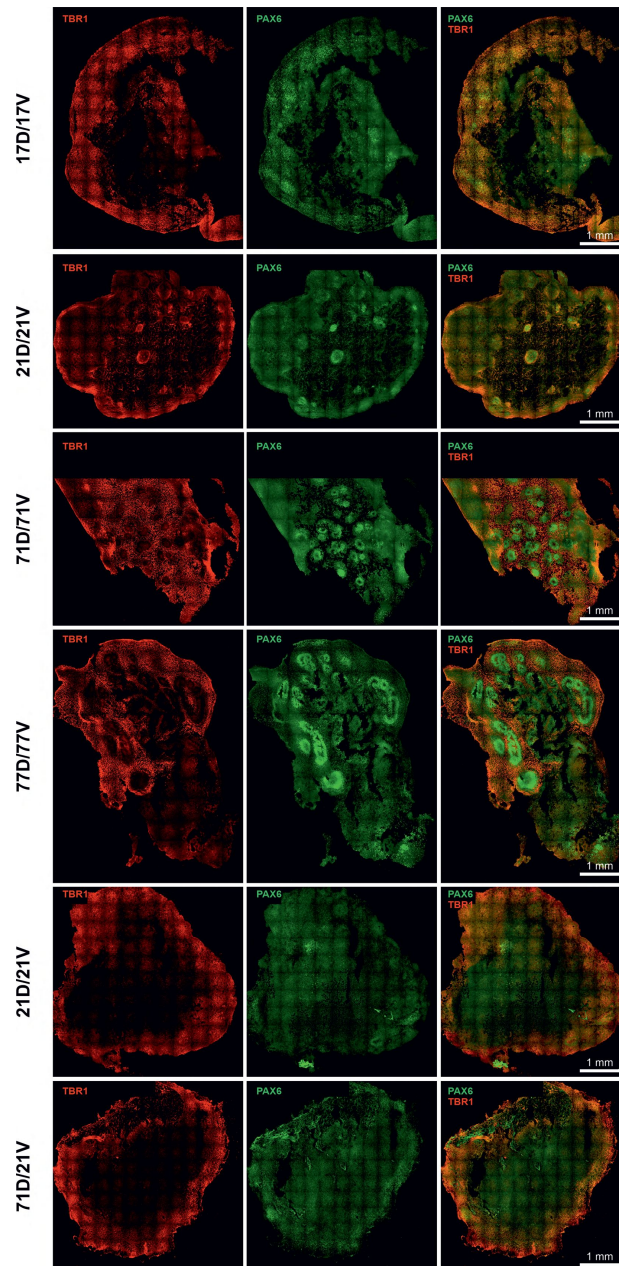


Figure 3.

Prominent occurrence of neuronal rosettes containing TBR1 and PAX6 positive neural stem cell populations in 60-day-old fused dorso-ventral HD organoids

The internal structure of the organoids and characteristic PAX6-positive (early progenitors, NSC) and TBR-positive (early-born neurons) dorsal populations in organoids were detected by immunostaining protocol. The panels of representative micrographs are designated by HD (71D/71V, 77D/77V), control (17D/17V, 21D/21V), and mosaic organoid (21D/21V, 71D/21V) types. The panels contain a co-staining experiment with PAX6 (green) and TBR1 (red) and a merged image of both stainings for each organoid. The micrographs revealed the occurrence of a large number of PAX6-positive neural rosettes lying close to each other and the occurrence of the separate layers of TBR1 expression in HD fused organoids (71D/71V & 77D/77V) while in control fused organoids (17D/17V & 21D/21V) only several scattered rosettes and lower expression of PAX6 and TBR1 is present, indicating less neural stem cells therefore less proliferative profile. In turn, HD organoids are directed towards the proliferative phase characteristic for early neural development with a vast number of progenitors in rosettes. Importantly, in the mosaic organoids, there is a regression in the HD phenotype represented by reduced neural rosette occurrence.

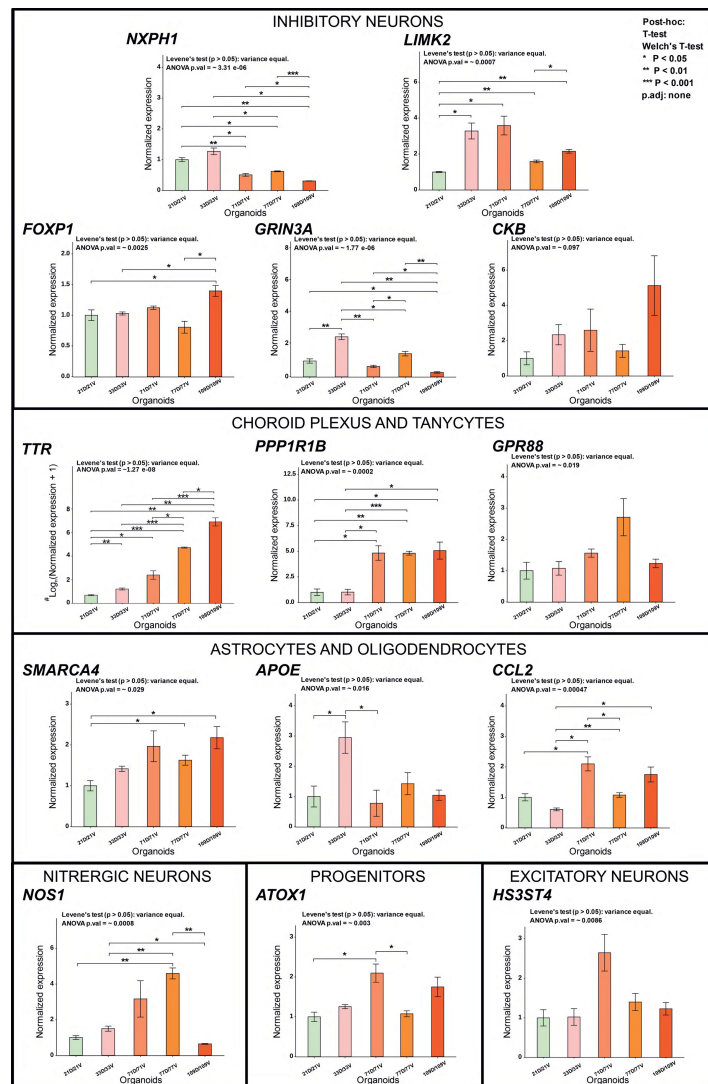


Figure 4.

Evaluation of the mRNA expression levels of neurodevelopmental marker genes of selected types of neuronal and non-neuronal cell populations in 60-day-old juvenile HD fused brain organoids.

The panels are designated with names of cell types and show graphs of mRNA levels of marker genes in 60-day-old fused dorso-ventral HD and control organoids originating from several iPSC lines. The graphs of marker genes are grouped by Inhibitory neurons, Choroid plexus and tanycytes, Astrocytes and oligodendrocytes, Nitroergic neurons, Progenitors and Excitatory neurons. The expression level of an analyzed gene in each type of fused organoid was compared to 21Q control organoids as basal expression level. The mRNA expression was normalized using *ACTB* as a reference gene. (Levene's test $p > 0.05$: was used for variance examination).

markers. *TTR* and *PPP1R1B* showed the most prominent upregulation in all HD lines compared to 21Q and 33Q control organoids. The changes in expression of the *TTR* mRNA were particularly differentiating across HD lines, with the increasing CAG lengths reaching 11.55 FC for 71D/71V, over 100 FC 77D/77V, and well over 1000 FC all vs 21D/21V control organoids. The 33D/33V organoids showed a 2.37 FC increase in *TTR* mRNA level vs. 21D/21V. The 33 CAG repeat line is still regarded as the healthy control; however, they contain a number of repeats that may indicate a certain risk of occurring some HD phenotypes (Fig 4 [↗](#)). ANOVA *p* val. ~ 0.00036; posthoc: T-test * *p*<0.05, ***p*<0.01, ****p*<0.001). *DARPP-32*, encoded by the *PPP1R1B* gene, plays a pivotal role in integrating signal transduction in dopaminergic neurons and has a regulatory role in the central nervous system (CNS) (Lin et al., 2021 [↗](#)). Another tanycyte marker, *GPR88*, showed elevated expression levels in the 77Q HD line compared to 21Q and 33Q control organoids (ANOVA *p* val. = 0.0196). In the group of astrocytes and oligodendrocytes markers, only one gene exhibited upregulation in 109Q HD organoids – *SMARCA4* (ANOVA *p*val = 0.0290). The expression of *ATOX1*, a marker of neuronal progenitors, and *HS3ST4*, a marker of excitatory neurons, was also increased in 71Q HD lines in comparison to 21Q and 33Q control organoids (ANOVA *p*val = 0.0033 and 0.0086, respectively). *CCL2* gene expression demonstrated downregulation in 71Q and 77Q organoids but a slight upregulation tendency in 109Q organoids (ANOVA = 0.0005). The opposite result was established for *NOS1*, a marker of nitrergic neurons, which showed upregulation in 71Q and 77Q organoids and downregulation in the 109Q line (ANOVA < 0.0008). All post-hoc test *p* values are presented in Fig. 4 [↗](#).

2.4. Pooled cell transcriptomes show early-developmental gene expression and GSEA profiles in Juvenile HD organoids and revers in mosaic juvenile HD/Control

Data from scRNA-seq were first used to identify the total number of DEGs and determine the main processes involved in HD and development in our organoids and mosaic organoids. We pooled cell transcriptomes based on genotypes and compared HD fused organoids (71D/71V, 77D/77V) vs. control (WT) fused organoids (17D/17V, 21D/21V), and mosaic HD/WT fused organoids (71D/21V, 21D/71V) vs. control fused organoids (21D/21V).

Obtained DEGs are presented as volcano plots (Fig. 5 [↗](#) A). We obtained 7815 downregulated genes and 123 upregulated genes for HD (71D/71V, 77D/77V) vs. control (17D/17V, 21D/21V) fused organoids, 9038 downregulated genes and 177 upregulated genes for mosaic HD/WT 71D/21V vs. healthy (21D/21V) organoids, and 8141 downregulated genes and 49 upregulated genes for mosaic WT/HD 21D/71V vs. control 21D/21V organoids.

Due to a certain variability of DEGs among organoids, we performed GSEA (see Materials and Methods section 2.8 [↗](#)). The terms that occurred most frequently for all organoids were manually assigned to arbitrary groups and presented on plots based on their *p*-value in supplementary data (Supp. Fig. 2,3 & Supp. 1,2,3,4,5).

The GSEA demonstrated that HD organoids exhibit upregulation of genes associated with terms tightly connected to early development and brain formation (mostly Dentate gyrus, Forebrain, Telencephalon, and Eye), also connected with epigenetics and Extracellular Matrix (ECM) formation [*H3-3A*, *BIRC5*, *RPS26*, *PAX6*, *LHX2*, *NR2E1*, *EMX2*, *HMG2*, *FEZF2*, *COL3A1*, *COL1A2*, *COL4A6*, *COL21A*, *VCAM1*, *TTR*, *LTBP3*]. In contrast, the terms enriched for downregulated genes in HD organoids are linked to processes characteristic of later stages of brain development, such as neuronal and glial cells maturation, migration, and signaling release related to processes of autophagy, mitotic spindle orientation and cilia motility. (Supp. Fig. 2A). Such gene deregulations and GSEA profiles indicate that control organoids are more related to maturation processes than HD organoids. Moreover, the profile of terms indicates that HD organoids delay or even arrest their development, preserving the stemness function of the cells and showing less relation to terms indicating brain structure development compared to healthy organoids. It can also be

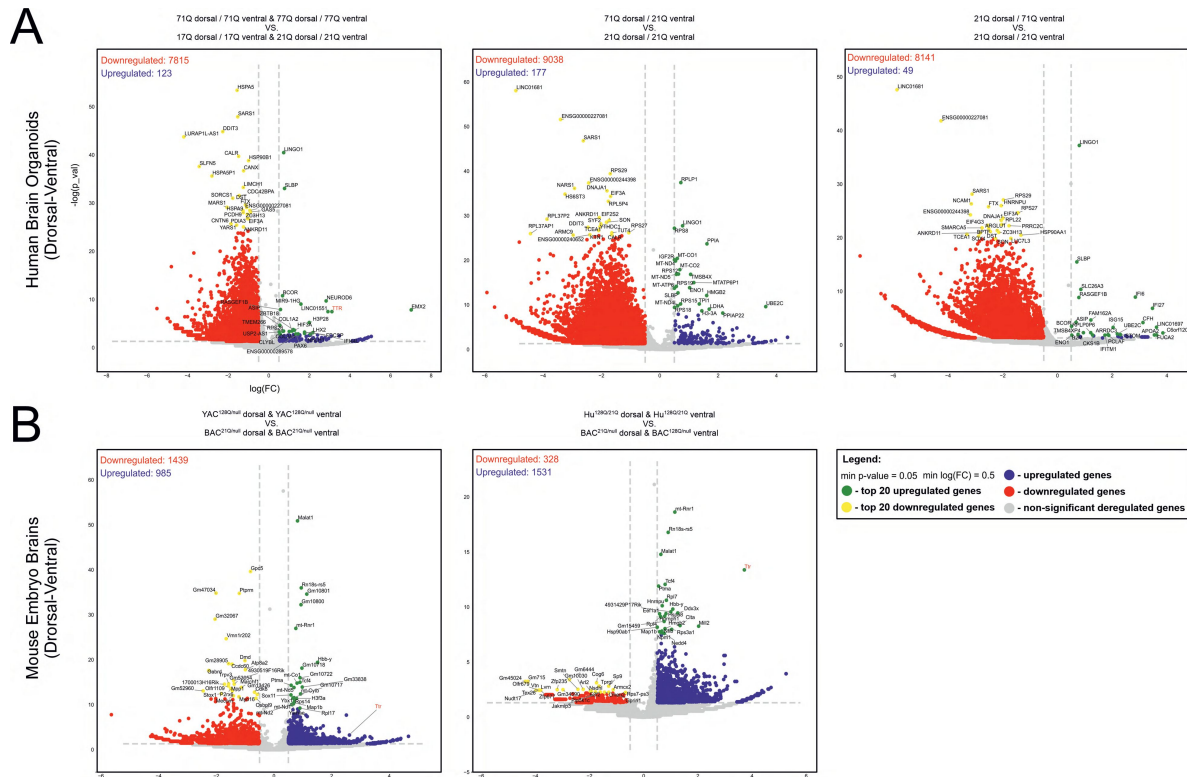


Figure 5.

Identification of deregulated expression of genes in pooled cell transcriptomes of juvenile HD, control and mosaic juvenile HD/control organoids, and HD embryonic brains.

The cell transcriptomes from scRNAseq experiments were first pooled by genotypes indicated on volcano plots, namely by types of organoids and ventral or dorsal parts of HD embryonic brains, (YAC^{128/null} and Hu^{128/21}) or control brains (Bac^{21/null}) for screening of DEGs in these datasets. A) The analysis of DEGs for organoids has shown the most striking upregulation of the *TTR* expression in HD organoids, which is closely related to the occurrence of the ChP population. This deregulation was not significant in both mosaic organoids. B) The analysis of mouse embryonic brains has shown upregulation of the *TTR* in both models (YAC^{128/null} and Hu^{128/21}). The analysis is extended by GSEA [Gene Set Enrichment Analysis], available as supplementary figures 2 and 3.

strongly related to alteration in such processes as mitotic spindle, cell polarity, cilia, cell motility, and autophagy, which play a crucial role in the early stages of healthy brain development. This is reflected by upregulated expression of specific gene markers such as *EMX2* [pval = $\sim 1.46\text{E-}08$; log(FC) = ~ 7.02] and *PAX6* [pval = ~ 0.001 ; log(FC) = ~ 1.34], which are early markers indicating a high degree of cell stemness in HD organoids. Moreover, the genes essential for proper cilia function in neurogenesis such as *IFT88* [pval = $\sim 4.58\text{E-}07$; log(FC) = ~ -1.14], *ARL13B* [pval = ~ 0.02 ; log(FC) = ~ -0.43], *KIF3A* [pval = $\sim 6.62\text{E-}06$; log(FC) = ~ -0.65], and mitotic spindle *MAPT* [pval = $\sim 4.99\text{E-}11$; log(FC) = ~ -1.2] are downregulated.

Furthermore, our GSEA analysis revealed that several processes and terms observed for HD vs control organoids were not found or were reversed for mosaic organoids 71D/21V and 21D/71V vs. control organoids. For instance, terms characteristic for HD-upregulated genes related to initial brain formation were not found, and terms for HD downregulated genes related to neuronal and glial maturation and migration were now identified for upregulated genes in mosaic organoids. (Supp. Fig. 2 B & C; Supp. 1,2,3 for all genes, terms, and statistics). The observation indicates the importance of the interaction of ventral and dorsal regions in brain structure development in HD. Moreover, in our human organoids, both affected ventral and dorsal parts are necessary for the full manifestation of the HD phenotype.

2.5. Embryonic brains of HD mouse contain gene expression and GSEA profiles associated with HTT function

We pooled cell transcriptomes based on genotypes and compared mouse embryonic brains from HD Yac^{128Q/null} vs control Bac^{21Q/null} and HD Hu^{128Q/21Q} vs control Bac^{21Q} (Fig. 5 B). We obtained 328 downregulated and 1531 upregulated DEGs for disease YAC^{128Q/null} vs. control BAC^{21Q/null}, and 1439 downregulated and 985 upregulated DEGs for Hu^{128Q/21Q} vs. control BAC^{21Q/null} embryonic brains.

The analysis of YAC^{128Q/null} vs BAC^{21Q/null} demonstrated downregulated genes mainly related to neuronal and glial cell proliferation, development, and regulation of cell signal release, autophagy, and ECM formation. For upregulated genes, we noticed processes related to embryo development and brain structure formation, regulation of neurogenesis, cell migration, myelination, and cell connection formation and signaling related to processes of mitotic spindle orientation, cell polarity, cilia, and programmed cell death (Supp. Fig. 3 A; Supp. 4).

Similar results were present in Hu^{128Q/21Q} vs BAC^{21Q/null} analysis except that downregulated genes also included terms and processes such as mitotic spindle orientation, cell polarity, and motility, and an increased number of genes assigned to cell maturation and single release terms. The upregulated genes demonstrated similar results to YAC^{128Q/null} vs BAC^{21Q/null} analysis (Supp. Fig. 3 B; Supp. 5).

In addition, the results of both YAC^{128Q/null} and Hu^{128Q/21Q} mouse embryonic brains demonstrated several upregulated genes related to the control of mitotic spindle, potentially indicating the occurrence of altered development and neurogenesis in HD embryos. The upregulated genes included *Map1B* [YAC^{128Q/null}: pval = $\sim 1.77\text{E-}08$; log(FC) = ~ 0.59 , Hu^{128Q/21Q}: pval = $\sim 3.47\text{E-}11$; log(FC) = ~ 0.69], *Clta* [YAC^{128Q/null}: pval = $\sim 4.83\text{E-}09$; log(FC) = ~ 1.34 , Hu^{128Q/21Q}: pval = ~ 0.00004 ; log(FC) = ~ 1.02].

2.6. Increased *TTR* mRNA level is a common feature of HD organoids and HD embryonic brains compared to control, mosaic organoids, and control brains

Analyzing all our HD organoid and HD embryonic models, we found that the *TTR* expression vastly increased in all HD models compared to their control counterparts, demonstrating high statistical significance. Pooled cell transcriptomes of 71D/71V & 77D/77V organoids showed *TTR* expression $\log(\text{FC}) = \sim 8.64$ [pval = $3.41\text{E-}08$] vs Control organoids (**Fig. 5** [A](#)); pooled cell transcriptomes of YAC^{128/null} embryonic brain showed *TTR* expression $\log(\text{FC}) = \sim 13.15$ [pval = $6.03\text{E-}23$], and Hu^{128/21} showed *TTR* expression $\log(\text{FC}) = \sim 5.70$ [pval = $2.1\text{E-}08$] all vs control BAC^{21/null} embryonic brain (**Fig. 5** [B](#)). Surprisingly, we found that our compensatory model, mosaic organoids in both HD ventral/control dorsal and control ventral/HD dorsal configuration compared to the control 21D/21V organoids did not reveal statistically significant changes in *TTR* expression (**Fig. 5** [A](#)).

TTR protein often occurs as deposits of fibrils in ECM in neurodegenerative diseases. Interestingly, the ECM formation genes firmly belonged to the downregulated gene pool in the HD embryo brain, and the same term was located in the upregulated gene pool in HD organoids. This could be attributed to the higher demand for extracellular matrix as a scaffold for cell proliferation in organoids, isolated model systems that are not very richly supplied with ECM compared to regular tissue such as the brain. In the tissue, ECM demand is provided by various cell types and is more balanced.

2.7. HD organoids and embryonic brains contain a prominent increase in choroid plexus cells, which are not present or diminished in mosaic organoids

We performed a droplet-based scRNA-seq of 60-day-old fused dorso-ventral JOHD (71Q dorsal / 71Q ventral and 77Q dorsal / 77Q ventral) and control (17Q dorsal / 17Q ventral and 21Q dorsal / 21Q ventral) organoids where we profiled approximately 8.000 single cells, and mosaic organoids (71Q dorsal / 21Q ventral and 21Q dorsal / 71Q ventral) with approximately 3.000 single cells. Additionally, we performed a scRNA-seq sequencing of mouse embryonic brains (e.13.5) from HD disease YAC^{128Q/null} and Hu^{128Q/21Q}, and control BAC^{21Q/null} model where we profiled 9.000 single cells.

We distinguished nine general cell populations from all analyzed organoids and embryonic brains characteristic for STR, CTX, MGE, LGE, caudal ganglionic eminence (CGE), hippocamp (HIP), tectum (TET), MEL, and ChP. Annotation of clusters to reveal population identities was performed as described previously (Kubiś and Figiel, 2023 [A](#)). Thanks to that, we were able to “zoom in” our results and divide general populations into many different subpopulations, and establish the cell lineage composition of the juvenile HD organoids and control organoids (Supp. Fig. 4) and also mouse embryonic HD and normal dorsal and ventral part of brains (Supp. Fig. 5).

The analysis of the single-cell data from organoids (**Fig. 6** [A](#)) has shown that there was no ChP population in the 17D/17QV and less than 1% in 21D/21V control organoids. In 71D/71V and 77D/77V fused organoids, the choroid plexus accounted for as much as 10% and 16% of all cells. Another cellular population, present only to HD organoids were melanocytes, which accounted for more than 2% and 4% in 71D/71V and 77D/77V fused organoids, respectively. Our scRNA-seq data show that there is a tendency in HD that more pluripotential cells are directed to develop the dorsal patterns (CTX, HIP, ChP, MEL (Visual Cortex)) in the direction of the cortical cells, than the ventral patterned cells (MGE, LGE, STR). We observed that the presence of ventral and dorsal cell types is imbalanced in mosaic organoids. For the 71D/21V mosaic organoids, where the ventral

side is healthy we noticed that the contribution of ventral part cells (MGE [17.19%], CGE [3.33%], LGE [0.6%]) is higher than dorsal cells (CTX [10.63%], ChP [2.24%], HIP [1.49%]). We observed the opposite in 21D/71V mosaic organoids, where the dorsal side is healthy and the ventral was affected. In this case, the share of dorsal part cells (CTX [24.32%], MEL [7.67%], HIP [1.86%]) is higher than ventral cells (MGE [0.88%], LGE [0.77%]). In both mosaic organoids, the share of STR ventral cells was on the same level ~64%.

scRNA-seq from mouse embryo brains (**Fig. 7**) has shown that dorsal brain part BAC^{21Q/null} revealed no Choroid plexus populations. Choroid plexus populations were a large fraction of dorsal embryonic brain parts of the YAC^{128Q/null} (49.64%) and Hu^{128Q/21Q} (35.78%) of all cells analyzed. In Hu^{128Q/21Q} we also observed 6.92% of cell occurrence of the melanocyte population. In the ventral part of brains, we were able to see a more balanced population distribution in all mouse embryonic brain models. In YAC^{128Q/null} and Hu^{128Q/21Q} small fractions of melanocyte populations ~ 1% occurred.

2.8. Juvenile HD organoids and embryonic HD brains express mitotic spindle and ciliary genes in cell subtypes of ChP, CTX and HIP

We selected a set of DEGs involved in mitotic spindle stabilization (*EMX2*, *CLTA*, *MAP1B*, *MAPT*), cilia (*IFT88*, *ARL13B*, *KIF3A*), and *HTT*, which is essential for both molecular structures and their functionality. We found that (**Fig. 8A**) control organoids co-expressed these genes in the cell populations mostly belonging to STR, MGE, CGE and CTX, while HD organoids co-expressed the genes mostly in ChP and CTX cell populations. In mosaic organoids, we have noticed co-expression of these genes in STR, MGE, MEL (melanocytes) cell populations and one cell subtype of ChP. Moreover, HD organoids reveal a high level of *EMX2* expression in many cell populations, compared to cell populations in control and mosaic organoids, where expression of *EMX2* is marginal.

In the healthy dorsal part of the mouse embryonic brain, we observed spindle- and cilia-related genes co-expression in populations of CTX, MGE, and HIP. In both HD dorsal brains (**Fig. 8B**), co-expression of these genes is primarily found in CTX, ChP, and HIP cell populations. In the case of the ventral part of the embryonic brain (**Fig. 8C**), in the control model, co-expression of these genes is predominantly observed in STR, LGE, MGE, CGE, CTX, and HIP. In the dorsal HD brain, there is a higher occurrence of co-expression with genes related to cilia, such as *Itf88*, *Arl13B*, and *Kif3A*, compared to the control model. Additionally, the *Htt* gene appears to be more frequently co-expressed in cell populations on the ventral brain side, whereas *Emx2* is more frequent for the dorsal side of mouse embryonic brain. In this context, the ventral site, as the source of differentiating and migrating interneurons (Bagley et al., 2017; Birey et al., 2017), is crucial for developing the neocortical cell populations in the dorsal part of the brain and may contribute to the developmental HD pathogenesis.

2.9. Choroid plexus structures in HD organoids and increased TTR Protein in blood serum of HD Mouse Model

We performed immunostaining for TTR in 60-days old fused dorso-ventral 21Q/71Q mosaic organoids and 21Q dorso-ventral control organoid (**Fig. 9A**). The results showed an abundant signal for TTR in 71Q dorsal/21Q ventral mosaic organoid and no signal in 21Q dorso-ventral organoids, which corresponds to the scRNA-seq results. Transthyretin (TTR), also known as prealbumin, is a 55 kDa homotetrameric protein that transports thyroxine and retinol-binding protein (RBP) in the serum and cerebrospinal fluid. Since we found such a significant contribution of choroid plexus populations in HD and mosaic fused organoids, we investigated the levels of TTR protein as a marker candidate in the blood serum of HD mice (YAC^{128/null}) using Western blot

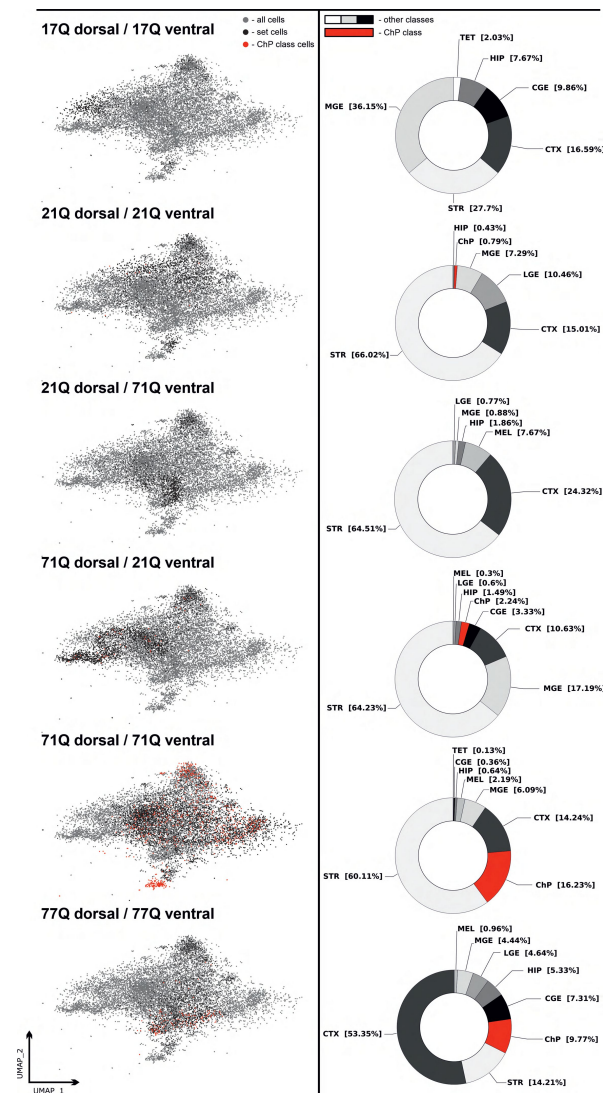


Figure. 6.

Indication of the main differences in all cell populations for HD (71Q dorsal/71Q ventral; 77Q dorsal/77Q ventral), control (17Q dorsal/17Q ventral; 21Q dorsal/21Q ventral), and mosaic (21Q dorsal/71Q ventral; 71Q dorsal/21Q ventral) organoids.

A) The UMAP presentation of the composition Choroid Plexus (ChP) population in HD, control, and mosaic organoids. There are noticed occurrence of ChP populations the most in HD (71Q dorsal/71Q ventral; 77Q dorsal/77Q ventral) and mosaic (71Q dorsal/21Q ventral) organoids. B) Percentage representation of cells belonging to ChP populations comparing to the rest populations in HD, control, and mosaic organoids. There are also noticed the occurrence of ChP populations the most in HD (71Q dorsal/71Q ventral; 77Q dorsal/77Q ventral) and mosaic (71Q dorsal/21Q ventral) organoids and less percent in control (21Q dorsal/21Q ventral) and complete lack of occurrence in control (17Q dorsal/17Q ventral) organoids.

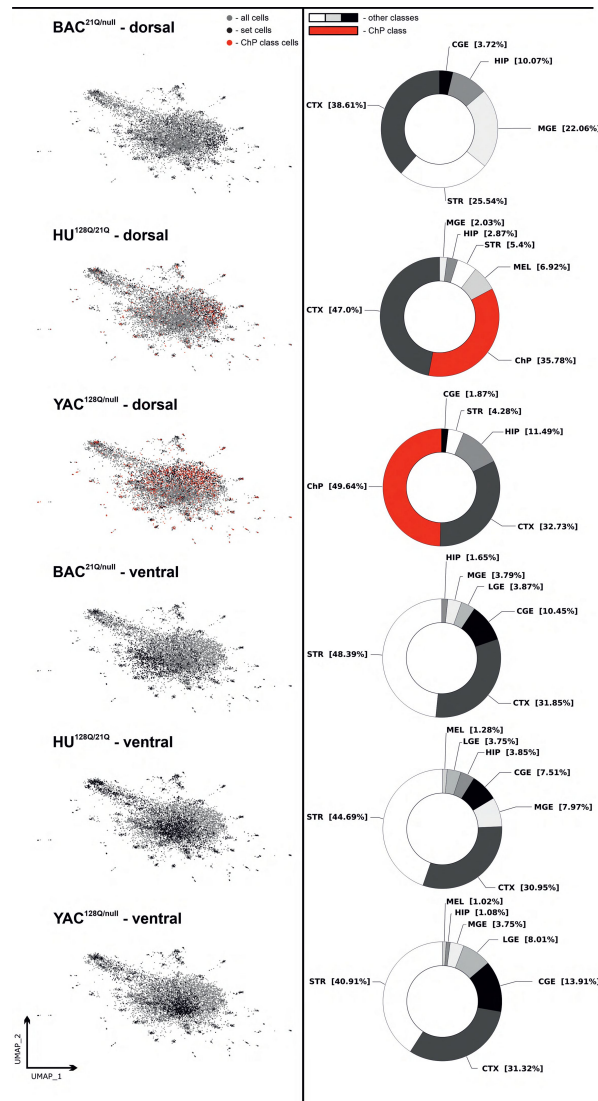


Figure. 7.

Indication of the main differences in all cell populations for full HD (YAC¹²⁸/null – dorsal & ventral brain), semi HD (Hu^{128/21} dorsal & ventral brain) and control (BAC²¹/null – dorsal & ventral brain) embryo brains.

A) The UMAP presentation of the composition of the Choroid Plexus (ChP) population in HD and control embryo brains. There are noticed occurrences of ChP populations just in HD cell populations (YAC¹²⁸/null – dorsal, Hu^{128/21} – dorsal) with dorsal lineages. B) Percentage representation of cells belonging to ChP populations compared to the rest populations in full HD, semi HD, and control embryo ventral and dorsal parts of brains. There are also approved occurrences of ChP populations only in HD (YAC¹²⁸/null – dorsal, Hu^{128/21} – dorsal) and a complete lack of occurrence in control dorsal and ventral brain parts (BAC²¹/null – dorsal & ventral brain) and also in ventral parts of HD brains (YAC¹²⁸/null – ventral, Hu^{128/21} – ventral).

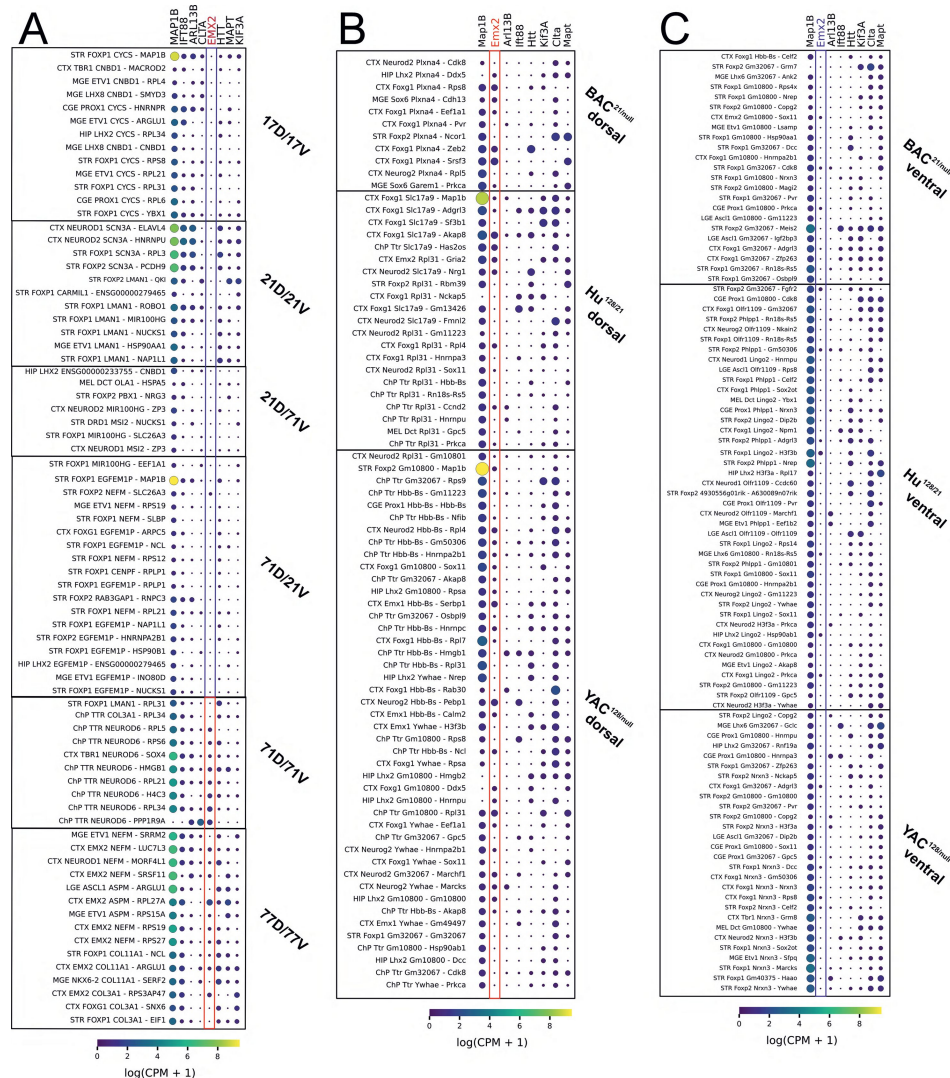


Figure 8.

In single-cell RNA sequencing (scRNAseq) data, we observed the co-expression of genes related to the mitotic spindle, cilia, and genes that regulate differentiation in selected cell populations across various datasets:

A) In the dataset for fused brain organoids, including healthy (17D/17V, 21D/21V), mosaic (17D/17V, 21D/21V), and disease (17D/17V, 21D/21V) conditions, we identified the co-expression of these genes, B) In the dataset for the dorsal part of mouse embryonic brains, encompassing healthy (BAC^{21/null}) and disease (Hu^{128/21} & YAC^{128/null}) conditions, these genes were found to be co-expressed, C) Similarly, in the dataset for the ventral part of mouse embryonic brains, including healthy (BAC^{21/null}) and disease (Hu^{128/21} & YAC^{128/null}) conditions, the co-expression of these genes was observed. These genes exhibited mutual expression across various cell populations in both normal and Huntington's disease (HD) affected brains. They are involved in essential processes such as differentiation, migration, and maturation, primarily through their influence on the mitotic spindle and cilia.

assay (**Fig. 9B**). Our findings show an increase in the level of TTR monomer (16 kDa) and tetramer (55 kDa) in YAC^{128/null} mice compared to control BAC^{21Q/null} mice (**Fig. 9**). The increased level of TTR protein corresponds directly with the scRNA-seq results of Hu^{128Q/21Q} mice and HD and mosaic fused organoids.

3. Discussion

The malformation or damage of the human brain during its neurodevelopmental phase can contribute to neurodegenerative disorders such as HD. Possible early neurodevelopmental processes that can be affected include aberrant neural stem cell proliferation, neuronal differentiation, and synapse formation (Mariani et al., 2015; McRae et al., 2017).

Brain organoids are rapidly evolving tools for understanding early human brain structure development and neurological diseases *in vitro*. Modeling of brain inter-regional interactions can be improved by directed differentiation of hiPSCs into distinct, region-specific organoids, which can later be fused, yielding a more faithful structural and functional, *in vitro* representation of the brain (Bagley et al., 2017; Birey et al., 2017).

An example of structure-dependent healthy brain function includes cerebral cortex layers that can activate striatal neurons through an organized glutamate-driven projection system, tightly regulated by dopamine (DA) and gamma-aminobutyric acid (GABA) (Rangel-Barajas and Rebec, 2016). Such precise interactions ensure appropriate motor and cognitive function of the cerebral cortex and the striatum. In a diseased HD brain, the glutamate, DA, and GABA neurotransmitter systems of the cortex and striatum are compromised. The flow of information is gradually destroyed between the structures, giving rise to both motor and cognitive impairments. The interaction between the striatum and cerebral cortex forms during early neurogenesis, and therefore, its malfunction in HD can be studied in brain organoids, which recapitulate early events in embryonic brain development. For instance, in fused organoids, interneurons generated in the ventral domain can migrate to the dorsal domain (Bagley et al., 2017; Birey et al., 2017), mirroring *in vivo* tangential migration patterns from the subpallium to the cerebral cortex (Anderson et al., 1997).

Interestingly, the structure that is formed in the vicinity of the cortex and striatum and affects neurogenesis is the choroid plexus. Its formation is a very early event timely correlated with neurogenesis. It begins with the invagination of mesenchymal cells into the neural tube early in gestation, forming a monolayer of cells with motility cilia close to inner cortex layers and striatum in brain ventricles (Gomez-Gamboa et al., 2014; Jain et al., 2010; Pala et al., 2017). The primary function of these ciliary cells is the secretion of CSF and growth factors, which combined influence play a crucial role in brain development by regulating the environment in which neurogenesis occurs (Bitanirwe et al., 2022; Johansson et al., 2013; Johansson, 2014; Lun et al., 2015b). This impacts neurogenesis, including progenitor survival, proliferation, and gliogenesis, in developing and adult mammalian brains (Moore and Iulianella, 2021).

The important function of the ChP-CSF system is the transport of molecules during critical periods of brain development. Along with choroid plexus, a very early event in development is the production and CSF secretion of transthyretin (TTR), a transporter of thyroid hormone and retinoic acid (Alshehri et al., 2020) (Alshehri et al., 2020; Bitanirwe et al., 2022; Johansson, 2014; Johansson et al., 2013; Lun et al., 2015b, 2015a). Importantly, the presence of mHTT influences abnormal motility cilia and causes abnormal CSF flow (Keryer et al., 2011). Noteworthy, enlarged volume of lateral ventricles in the brain is an early phenotype of adult and juvenile forms of Huntington's Disease (HD) (Hobbs et al., 2010; Jahanshahi et al., 2022). This phenotype may also correspond to possible extracellular ionic homeostasis dysregulation since ChP modulates the levels of various ions, such as sodium, potassium, and chloride, for maintaining

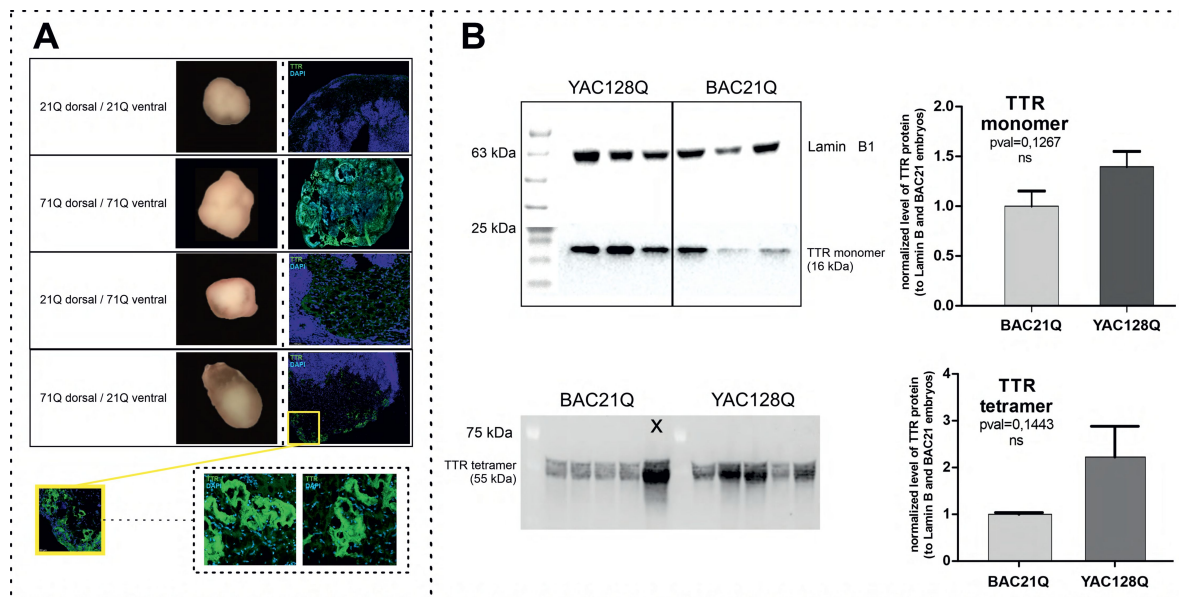


Figure. 9.

Molecular validation of scRNA-seq results of *TTR* upregulation and choroid plexus impairment results using immunofluorescence and Western blotting.

(A) Immunofluorescence staining for TTR in 60-day-old fused dorso-ventral 21Q/71Q mosaic organoids and 21Q dorso-ventral control organoids. IF results show an abundance of TTR in 71Q dorsal/ 21Q ventral mosaic organoids compared to the other organoids, which corresponds to the scRNA-seq results. (B) Western blot analyses of blood serum from adult 6-8-months mice show non-significant but yet an elevation level of TTR monomer and tetramer in YAC^{128Q/null} mice compared to BAC^{21Q/null} mice. The level of TTR monomer protein (16 kDa) was normalized to the level of the reference protein LAMIN B1. The graph was generated in GraphPad Prism. The statistical test used was the t-test for independent samples (n=3). (B) The level of TTR tetramer protein (55 kDa) was normalized to the total amount of protein in lanes stained with Ponceau S solution. The graph was generated in GraphPad. The statistical test used was the t-test for independent samples (n=5).

proper neural stem cell function in new neuron formation (Bitanirwe et al., 2022 [↗](#); Lun et al., 2015b [↗](#)). Therefore, it is plausible that the early enlargement of the ventricle phenotype may not be the primary result of brain degeneration but specific neurodevelopmental processes.

Our work aimed to discover early brain defects in HD and demonstrate that modeling the dorso-ventral forebrain axis as equivalent to cortico-striatal interactions is suitable for discovering such phenotypes in HD. We focused on early-rising cell populations that may be affected in the early phases of HD brain development using the fused dorsal and ventral forebrain organoids and HD mouse brains. Later, we examined HD phenotypes and cell populations when only one of the fused organoid parts, dorsal or ventral and vice versa, was HD or control (mosaic D/V and V/D fused organoids). Therefore, we have generated the HD and control organoids from iPSC lines by priming to the dorsal/neocortex (*TBR1* and *PAX6*) and ventral/striatum (*NKX2.1*, *DLX2*, and *GSX2*) identities. The dorsal/ventral fused organoids showed cortical and striatal cell markers and mature neurons.

We also noticed that, in general, fused HD organoids were growing significantly larger than the control organoids as measured by their 2D area, indicating the differential composition of cell populations in HD vs. control fused organoids. We hypothesized that the HD organoids have increased proliferative features compared to control fused organoids. Such proliferative responses may indicate increased proliferation of neural stem cells in HD organoids instead of neuronal differentiation. Previous work indicated a transcriptional immaturity in HD organoids relative to controls (Conforti et al., 2018 [↗](#)). Indeed, the dorsal part mimicking the developing forebrain of HD organoids demonstrated the abundance of *PAX6* positive neural rosettes, indicating the abundance of neural stem cells, surrounded by *TBR1* positive surroundings, indicating early-born neurons, while control fused organoids only scarcely contained stem cell rosettes. We also investigated our compensatory mosaic model, which showed few rosettes, a number similar to control organoids, indicating the reversal of the increased-rosette-number phenotype. Importantly, we observed an obvious morphological concrescence zone of cortical and striatal parts in HD organoids. Together, this shows that both parts of organoids may cooperate to exert HD phenotypes. This could indicate a cooperative influence of both cortex and striatum on HD neurodevelopmental deficits in HD patients.

The larger HD organoid size, the prominent occurrence of neural stem cell rosettes, and their concomitant influence on the striatal and dorsal part may be the result of changes in specific cellular populations of HD organoids. We first selected genetic markers of cell populations in the developing brain where *HTT* expression was present at high levels or completely absent (using public scRNA-seq data). Our findings revealed significant deregulation in several inhibitory neuron markers associated with the neuropathology of AD and autism (Cuberos et al., 2015 [↗](#); Yang and Shcheglovitov, 2020 [↗](#)) and downregulation of synaptogenesis and synaptic integrity markers (Saykin et al., 2010 [↗](#)).

The most striking result across all HD lines was the 10 to 1000 fold and highly consistent upregulation of markers lying at the interface of cortex and striatum, namely choroid plexus markers, *TTR*, and high level of dopaminergic striatal neuronal cell marker *PPP1R1B*. Other markers with elevated expression across organoid HD samples were *GPR88*, which has been linked to various psychiatric disorders (Massart et al., 2016 [↗](#)) and astrocytes and oligodendrocytes marker, *SMARCA4* deregulated in HD mice (Capizzi et al., 2022 [↗](#)).

Next, to better understand the cell composition and cellular phenotypes in HD, we used our fused HD brain organoids for scRNAseq. Further, we aimed to investigate the role of dorsal and ventral brain organoid parts in HD by using our compensatory model, fused mosaic HD/Control organoid, and alone dorsal and ventral brain parts dissected from HD and control embryos. Moreover, we

selected the E13 embryo developmental stage, which is just adjacent to the beginning of the accelerating phase of choroid plexus development in mice and associated with expansion with the beginning of neurogenesis (Fame and Lehtinen, 2020 [DOI](#)).

Cell transcriptomes of scRNAseq were first pooled based on genotypes for the identification of DEGs and the main processes involved in juvenile HD neurodevelopment. Analysis of HD vs control organoids has shown mainly downregulation in crucial processes for brain development regulation, such as mitotic spindle, cell polarity, cilia, cell motility, programmed cell death, and neuronal differentiation and maturation. This indicates that spindle orientation and ciliogenesis, and symmetrical or asymmetrical cell divisions crucial of cortical layers formations which are, in fact, HTT-dependent, can be disturbed in HD organoids (di Pietro et al., 2016 [DOI](#); Godin and Humbert, 2011 [DOI](#); Guemez-Gamboa et al., 2014 [DOI](#); Keryer et al., 2011 [DOI](#); Mukhtar and Taylor, 2018 [DOI](#); Pala et al., 2017 [DOI](#); Park et al., 2019 [DOI](#)). In turn, the upregulated processes were related to the regulation of neurogenesis, brain structure development, epigenetic regulations, and ECM formation, which are connected to the reprogramming and proliferation of NSC, hence the phase before neuronal differentiation. Again, we observed a reversal in our compensatory mosaic model (HD/control) concerning the main gene deregulations related to biological terms (Supp. Fig. 3 B & C). The processes and gene deregulations are closely related to an upregulation of genes *ESCO2*, *BIRC5*, and *PAX6*, related to mitotic spindle stabilization, and very high expression of *EMX2*, which in cortical development promotes clonal expansion and symmetric cell divisions (Mukhtar and Taylor, 2018 [DOI](#)). This demonstrates a strong response of developing HD organoids to the influence of mHTT on these processes and putative cellular attempts of activation of some compensatory pathways, likely activating the cell cycle control system, influencing pluripotency and differentiation (Aponte and Caicedo, 2017 [DOI](#); Liu et al., 2019 [DOI](#)). Again, the DEGs analysis has demonstrated high levels of TTR expression in HD organoids and a lack of upregulated expression in mosaic organoids.

Our flagship analysis of the single-cell transcriptomes defined the populations of HD organoids, mosaic organoids, and embryonic HD brains. We have detected that HD organoids were highly populated by TTR-positive choroid plexus cells, also characterized in our scRNAseq data by the expression of *TJP1* (pval = $\sim 2.54 \times 10^{-8}$; log(FC) = ~ 7.59) and *GATA3* transcription factor (pval = $\sim 1.93 \times 10^{-5}$; log(FC) = ~ 4.71), which is prominently active in the development and the *S100B* (pval = ~ 0.00048 ; log(FC) = ~ 3.13). In contrast, we observed a minimal or complete absence of ChP cells in control fused organoids. In mosaic organoids, we observed the ChP population in the 71D/21V mosaic, whereas it was absent in the 21D/71V mosaic. Similar to HD organoids, dorsal brain parts of YAC128/null and Hu128/21 were also highly populated with choroid plexus cells, while this was not the case for BAC21/null. None of the ventral parts were populated by choroid cells. These results may indicate that the timing of development of choroid plexus cells is different in HD vs control. Moreover, the abundant population of the choroid in HD corresponds to the upregulation of many NSC genes (eg. *PAX6* and *EMX2*, *TBR1*), indicating overactivated neurogenesis and less differentiation also in embryos. Probably, the overproduction of choroid cells also sustains until adulthood since a high level of TTR protein in CSF was reported in HD patients (Caron et al., 2022 [DOI](#)). Additionally, in mosaic organoids, when the dorsal part (containing some ChP) was affected, and the ventral part was a control, we observed a higher number of cells with more advanced differentiation characteristics of ventral brain regions (STR, LGE, MGE). Conversely, when the ventral part of the mosaic was affected, and the dorsal part was a control, we observed a higher number of populations of cells with characteristics of dorsal brain regions (CTX, HIP) but still no ChP population. In general, this demonstrates that the HD part is always delayed regarding its differentiation status.

To summarize our findings, we introduce novel organoid models for studying Huntington's disease and conduct an array of low and high-throughput analyses, revealing changes in HD development closely linked to the HTT protein's function. We elucidated the neurodevelopmental character of HD and demonstrated a developmental aberration consisting of an increased

occurrence of Choroid plexus and TTR expression, which is a very early developmental event in HD. The increased Choroid plexus affected the early developmental deficit in cell differentiation and correlated with increased abundance of NSC. TTR serves as an essential marker of the Choroid plexus. However, its increased levels also occur in the serum of HD animals. Our study highlights the potential of ChP biomarkers in blood serum as non-invasive markers for early detection and monitoring of HD. In addition, the Choroid plexus may represent a potential tissue of interest for novel therapeutic approaches, including detoxification by interference with the blood-brain barrier and regulation of growth factors and cell differentiation within the brain ventricles' microenvironment.

4. Materials and methods

All experiments were conducted in accordance with the relevant guidelines and established standards.

4.1. Human HD Induced-Pluripotent Stem Cells Culture

Human episomal HD and control iPSC lines were obtained from the NINDS Human Genetics Resource Center DNA and Cell Line Repository (<https://catalog.coriell.org/1/ninds>), from NINDS Human Cell and Data Repository (NHCDR) and from Cedars-Sinai Induced Pluripotent Stem Cell Core. For the analyses, we used three different HD lines with 71, 77 and 109 CAG repeats, and three control lines with 17/18, 21 and 33 CAG repeats. Human iPSCs were cultured in chemically defined conditions in Essential 8 medium (Life Technologies) and grown on Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Life Technologies). Cells were passaged using gentle dissociation with 0.5 mM EDTA in PBS.

4.2. Brain Organoid culture, differentiation and fusion

We developed the protocol for generating dorso-ventral forebrain organoids based on fusion protocol (Bagley et al., 2017; Knoblich et al., 2017) from human control and HD iPSC lines using an intermediate EZ spheres (neurospheres) culture step (Ebert et al., 2013). In brief, hiPSCs were detached with 0.5 mM EDTA in PBS, placed on a polyHEMA [Poly(2-hydroxyethyl methacrylate), Santa Cruz Biotechnology] coated dishes, and then allowed to form EZ spheres (Ebert et al., 2013) the next day. On the first day, EZ spheres were cultured in E8 medium with ROCK inhibitor (Y-2736, Tocris). On the second day, E8 medium were replaced with EZ medium (DMEM, F12, 2% B27 -vit. A, 1% penicillin/streptomycin, 0.5% Glutamax, 0.1% heparin) with growth factors, bFGF and EGF (ORF Genetics). After later mechanical passaging with McIlwain Tissue Chopper (model TC752, Campden Instruments) and obtaining the appropriate shape and diameter (>500 µm), neurospheres were incubated with small molecule inhibitors and activators of signaling pathways for dorsal (cyclopamine A: SHH inhibitor by smoothened inhibition) and ventral forebrain patterning (WNT inhibition by IWP2 and SHH activator by SAG agonist). Subsequently, the dorsal and ventral patterned organoids were fused and differentiated on the orbital shaker. Fused dorsal-ventral HD and control brain organoids, as well as our compensatory models - mosaic fused organoids (dorsal HD/ventral control and ventral HD/dorsal control), were collected on day 60 for scRNA-seq, RT-qPCR and immunohistochemistry analyses. Dorsal and ventral organoids from each cell line, which were cultured without the fusion step, were collected on day 60 for cortical and striatal markers assessment via qPCR.

4.3. Organoids Area Measurement

Pictures of 60-days old fused organoids were captured with Q-scope digital microscope model QS.80200-P (Euromex). Area measurement analysis was performed in ImageJ software using the "Freehand selection" tool. The scale was set based on the photograph of a calibration ruler added

to the Q-scope kit, with a known number of pixels equaling the length of 5 mm. Graphs were created using GraphPad Prism Software (Boston, MA, USA).

4.4. HD mouse tissue

The HD (Hu^{128Q/21Q}, Yac^{128Q/null}, Bac^{21Q/null}) mouse model was described previously (Southwell et al., 2017) and was maintained and bred in the Center for Advanced Technology (CAT) animal facility in Poznan. Animal experimentation and handling were approved and monitored by the Local Ethical Commission for Animal Experiments in Poznan (64/2018). For scRNA-seq analyses, pregnant female mice were sacrificed, embryos harvested, and embryonic brains dissected into cortex (dorsal part) and basal ganglia (ventral part). Blood serum for WB analyses was collected from the HD mouse model, from a typical experimental group of three or five mutant mice versus three or five control mice.

4.5. *In Silico* Analyses of Available Brain Region Transcriptomics Data

Public scRNA-seq datasets were acquired from NCBI GEO and Broad Institute Data Portal (<https://singlecell.broadinstitute.org/>) to select cell population markers in different brain structures for initial expression analyses. We used mouse brain datasets including hypothalamus [HYP] (7 329 cells; GSM3562050 and GSM3562051); embryonic thalamus (E12.5) [TH] (7 365 cells; GSE122012); motor cortex [MCTX] (13 539 cells; MD704, MD705); auditory cortex [ACTX] (35 594 cells; MD717, MD720, MD21); orbitofrontal cortex [OCTX] (19 531 cells; MD716, MD717); entorhinal cortex [ECTX] (80 653 cells; MD720, MD721, MD698/699); hippocampus [HIP] (6 555 cells; MD718, MD719) and cerebellum [CER] (611 034 cells). The datasets represented both single-cell and single-nucleus approaches; therefore, all mitochondrial and metabolism-related genes were excluded from the analysis based on genes from MitoCarta 2.0. Data from each mouse brain region were analyzed separately using our original JSEQ_scRNAseq pipeline designed to discover the composition of cell populations in high resolution (https://github.com/jkubis96/JSEQ_scRNAseq).

The cell populations from the high-resolution analysis were next analyzed using statistical and machine learning methods, such as a decision tree (rpart), correlation (corr), and statistical cell population comparison using the Shapiro-Wilk, T-Student, and Mann-Whitney tests with differences in the expression level of marker genes on significant level $p \leq 0.05$. Subsequently, the specific cell population (e.g.: GABAergic neurons) was separated using quantiles 1 and 3 for high and low *HTT* gene expression in particular cells. The populations were then analyzed to obtain high expression of their specific markers and high *HTT* expression (R software with RStudio IDE). Specific qPCR primers (Table 1) were designed for each marker gene using MEGAX, Primer3, and NCBI primer blast tools.

The preanalyzed data used for this preliminary selection of markers are available and have been described (Kubiś and Figiel, 2023).

4.6. scRNA-seq

We performed scRNA-seq using cells from 60-days-old entire fused brain organoids or from E13.5 mouse embryonic brains dissected into dorsal (containing embryonic CTX, HIP, ChP) and ventral (containing embryonic STR, HYP, TH) parts. The day 60 organoids and dissected dorsal and ventral embryonic brains were incubated with 0.5 ml TrypLE (Thermo Fisher Scientific) for 5 minutes at 37°C with agitation (orbital shaker; 60 RPM), followed by gentle pipette trituration, further incubation for 3 minutes followed by another gentle trituration for 10 times. TrypLE was inactivated by diluting the sample with 1 mL of PBS-EDTA with 0.01% BSA, followed by centrifuging for 3 min at 300 x g. The cells were resuspended in 1 ml of PBS-EDTA with 0.01% BSA,

Gene symbol	Forward primer sequence	Reverse primer sequence
<i>PAX6</i>	TGGGCAGGTATTACGAGACTG	ACTCCCGCTTATACTGGGCTA
<i>TBR1</i>	GCAGCAGCTACCCACATTCA	AGGTTGTCAGTGGTCGAGATA
<i>DLX2</i>	ATGCACTCGACCCAGATCG	GGCTTGGTACTGGTAGGAACC
<i>GSX2</i>	ATGTCGCGCTCCTTCTATGTC	CAAGCGGGATGAAGAAATCCG
<i>NKX2.1</i>	ATGAAGCGCCAGGCTAAGG	GGTTTGCCGTCTTTGACTAGG
<i>TTR</i>	TGGGAGCCATTTGCCTCTG	AGCCGTGGTGGGAATAGGAGTA
<i>CCL2</i>	CCCCAGTCACCTGCTGTTAT	AGATCTCCTTGGCCACAATG
<i>LIMK2</i>	GAATCTGGAGGGGACACTGA	ACCTCCCCATGGATTAGGTC
<i>FOXP1</i>	ATCCCGATGTGTCTGTGTGA	GCAGTTGCACAAAAAGCAAA
<i>MSTO1</i>	ATGGTGTCTGGAGGGTCAAA	GGGATGGAGATGGACTCTGA
<i>ATOX1</i>	AGGCTGTGCTGAAGCTGTCT	TCCATGCTGTGCTCAGATTC
<i>CKB</i>	ACGACGTCATCCAGACAGG	AGGTCGGTCTTGTGCTCATC
<i>APOE</i>	GGTCGCTTTTGGGATTACCT	TCCAGTTCCGATTTGTAGGC
<i>GRIN3A</i>	CCACTCCACTGGACAATGTG	GGTTTGATGGCCACTGTTCT
<i>NXPH1</i>	GAGCAAGACCTCTGGGACTG	AAATGTCCCATTGCCATGAT
<i>NOS1</i>	TTGAAAAGGCCAACAATTCC	TTGACCGACTGGATTTAGGG
<i>GPR88</i>	CCCGGTGTCACTCCTGTATT	GTTACGATGAAGGCGTTG
<i>PPP1R1B</i>	CAGAGGAGGAGGATGAGCTG	AGCAGACTGCCTGATGACCT
<i>HS3ST4</i>	CATCATCGGGGTCAAGAAAG	CACTCCAACCCCTTTTCGTA

Table 1.

Sequences of primers used for qRT-PCR analyses.

with few pipetting actions, and passed through a 40 µm cell strainer. The cell number was determined using Trypan Blue and manual counting under a light microscope. A suspension of 300,000 cells/mL was prepared from the initial cell suspension using PBS-EDTA with 0.01% BSA.

Encapsulation of cells and further processing were prepared according to the protocol outlined in the Nadia source publication (Davey et al., 2021 [DOI](#)). In brief, the cell suspension (75,000 cells) was encapsulated with the barcoded beads (150,000 beads) using Nadia Instrument (DO BIO, UK) and disposable microfluidic cartridges, according to the pre-programmed protocol for drop-seq available in the instrument. The droplet beads undergone multiple purification steps using Überstrainer and were washed with 6 x SSC, Tris-EDTA (TE), and Tween solutions to remove the oil remnants from the beads. Subsequently, the beads with captured RNA were reverse transcribed (Maxima RT Fisher Scientific, incubation 30 min RT + 90min 42°C), and the unused barcodes were removed from the beads with exonuclease I (NEB, incubation 45min 37°C), according the Nadia protocol (Davey et al., 2021 [DOI](#)). For 3' mRNA profiling, cDNA libraries were amplified using a PCR reaction (2,000 beads per reaction) with Kapa HiFi Mix (Roche) and Smart PCR Primer, employing the previously described protocol (Davey et al., 2021 [DOI](#)) and (Macosko et al., 2015 [DOI](#)) with modifications of the PCR cycle extending up to 15 cycles. The amplified cDNA was purified by Ampur XP beads and the concentrations were estimated using Qbit assay. Subsequently, libraries for sequencing (NextSeq550 platform) were prepared using the Nextera XT/Vazyme kits and protocols (Illumina/Vazyme), which was modified after the tagmentation step by adding number of cycles up to 18. For library amplification, custom Index2 (I5) was used to select only tagmentation products that included UMI and BARCODE sequences (Davey et al., 2021 [DOI](#)) and (Macosko et al., 2015 [DOI](#)). The library size was adjusted with Ampur XP beads, and its quality and quantity was checked using a quantity control via the Qubit assay and a quality control via the TapeStation System. Sequencing was performed on the Illumina NextSeq550 platform using the NextSeq 500/550 High Output Kit v2.5 (150 Cycles). The sequencing depth was adjusted to around 10,000 reads per cell, which is sufficient for general single-cell characteristics concerning the count of organoids and mouse embryonic brains sequenced.

4.7. Bioinformatic Analysis of scRNA-seq Data for rare cell populations

Raw FASTQ reads collected from NextSeq550 were analyzed by JSEQ_scRNAseq v.2.3.2 bioinformatics pipeline, which was previously described (Kubiś and Figiel, 2023 [DOI](#)) and is available at https://github.com/jkubis96/JSEQ_scRNAseq. In brief, the pipeline included read quality control (FASTP), annotation to human GRCh38.p13 genome in case of organoids data or mouse GRCm38.p6 genome in case of mouse embryonic brain regions. Further pipeline steps included UMI and BARCODES quality control and repair (UMI-tools, DropSeq), data clustering with markers selection (Seurat), and other additional steps previously described in detail (Kubiś and Figiel, 2023 [DOI](#)) for final production of high-resolution data including rare cell populations occurring in organoids and embryonic brains. The cell populations were also annotated to their biological functions. Analyses were conducted with default settings included in JSEQ_scRNAseq pipeline and described in Manual available on GitHub and in the publication. The analyses for every single organoid type (HD, control and mosaic) and mouse embryonic brain regions were conducted separately to obtain higher data resolution.

Downstream analysis of obtained cell clusters from JSEQ_scRNAseq results was conducted using a Python script (Supp. 6) with Spyder IDE. The Python scripts were used for joining data from HD vs. normal vs. mosaic organoids and HD vs. normal embryonic mouse brain regions. The analysis included Harmony adjustment, selection of inter-set and intra-set marker genes, visualization of cell populations from different data sets on UMAPs with Harmony adjustment, and visualization of single-cell data composition (pie plot, bar plot).

4.8. Identification of the differentially expressed genes (DEGs) and GSEA processes from pooled cell transcriptomes

The scRNA-seq cell transcriptomes were initially pooled based on genotypes and used to identify the total number of DEGs for HD vs control and mosaic vs control for organoids and YAC^{128Q/null} vs BAC^{21Q} and Hu^{128Q/21Q} vs BAC^{21Q} for embryonic brains. The DEGs were selected by p-value ≤ 0.05 with Mann-Whitney and minimal log(FC) ≥ 0.5 and visualized on volcano plot of disease vs. healthy data sets. The identified DEGs were then used for Gene Set Enrichment Analysis (GSEA) performed with GEDSpy (default settings) python library available at <https://github.com/jkubis96/GEDSpy> with Spyder IDE. The gene enrichment was conducted for GO-TERMS and PATHWAYS (REACTOME, KEGG). The GSEA was performed based on gene overrepresentation in biological terms with Fisher's Exact test and adjusted by Benjamini and Hochberg FDR correction. In the analysis, we selected only terms with adjusted pval ≤ 0.05 . The GSEA was performed separately for the upregulated pool and downregulated pool of genes. Subsequently, the terms identified by GSEA were analyzed to select the terms that differentially occur between downregulated vs upregulated gene pools by calculating a specific "term fold change". The "term fold change" is the term normalized by gene occurrence in upregulated or downregulated gene pool calculated as [number of genes related to terms divided by the number of genes inputted to analysis]. We set the term fold change cut off value as FC ≥ 1.5 . The biological terms showing the value of term fold change were then manually selected to only terms related to altered development and assigned to arbitrary groups of terms. The terms with the suitable term fold change and related to development identified from DEGs from organoids (HD, control) were then used as the core terms in other analyses in mosaic organoids and embryonic brains (YAC^{128Q/null}, Hu^{128Q/21Q}, BAC^{21Q/null}). The entire GEDSpy analysis output is available in supplementary data.

4.9. RNA Isolation and RT-qPCR

After medium removal, HD forebrain organoids were washed once with PBS, transferred to 1,5 mL Eppendorf tubes, and then covered with 1 mL of TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instruction and frozen in -80°C . Upon thaw, total RNA isolation was performed according to the manufacturer's protocol with chloroform, isopropanol, 75% ethanol, and RNase-free water. Reverse transcription was performed using Maxima H Minus Reverse Transcriptase (Thermo Fisher) (200U per reaction) on 1 μg of RNA in 20 μL of total reaction according to the manufacturer's protocol. For priming a mixture of random hexamers (25 pmol) and oligo(dT) 18 (25 pmol) was used. Additionally RiboLock RNase inhibitor was added to the reaction mix (20U). Before adding the enzyme and the inhibitor, templates were denatured in 65°C for 5 minutes; after mixing all reaction reagents, the reaction was incubated for 10 min at 25°C followed by 15 min at 50°C . The resulting cDNA was further diluted to a final concentration of 10 ng/ μL and stored in -20°C . qPCR was performed on CFX96 Real-Time System (Bio-Rad) using HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (SOLIS BIODYNE) on 1 μL of cDNA in 10 μL of total reaction volume. The reaction mix included 250/125 nM primers. All primers are listed in **Table 1**. Thermocycling parameters were as follows: 15 min of initial denaturation at 95°C and 39 two-step cycles with 15 s denaturation at 95°C and 60 s annealing at 60°C , followed by 5 s melt curve from 65 to 95°C , increment $0,5^{\circ}\text{C}$. The reaction was run on CFX96 instrument (Bio-Rad). The data were analyzed, and Cq values were determined using a regression model by CFX Manager 3.1 (Bio-Rad). ACT β was used as a reference gene.

4.10. Immunohistochemistry

After medium removal, organoids were washed with PBS and fixed in cold 4% (vol/vol) paraformaldehyde (PFA) overnight at 4°C . Then, organoids were stained with Ponceau S for 20 minutes, incubated in 30% sucrose solution for 24-48 hours to protect from tissue damage during freezing, and flash-frozen in OCT Compound™ using dry ice. Flash-frozen organoids were stored in -80°C . Organoids were sectioned using Microtome cryostat (Leica) at -20°C . 25 μm sections were

collected at adhesive Superfrost Plus™ glass slides and air-dried at room temperature overnight. Then, the sections were blocked in 4% Normal Goat Serum (NGS) in TBS-T buffer for 1 h at room temperature. Primary antibodies were diluted in solution 4% NGS in TBS-T. All of primary antibodies used in this study are listed in **Table 2** [↗](#). Secondary antibodies were used in 1:500 dilution in 4% NGS in TBS-T. Hoechst 33258 was used to visualize nuclei. A series of fluorescent confocal pictures of human brain organoids were acquired using either the TCS SP5 II (Leica Microsystems; Poland) or Opera Phoenix Plus High-Content Screening System with x40 magnification, which required assembling images along the x-, y-, and z-axis. After using the Opera Screening System, the assembly operation was conducted using JIMG v.1.0.0., available at <https://github.com/jkubis96/JIMG/tree/v.1.0.0> [↗](#).

4.11. Western Blot Analysis

Fused organoids were harvested from 60-days old cultures. Blood samples were harvested from 6-8-month-old Yac^{128Q/null} and Bac^{21Q/null} mice. All tissues were homogenized in a phosphate buffer (0.1M, pH 7.5) with 2 mM PMSF using pipetting, followed by bath sonication for 10 min. The protein concentration was estimated using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. For each analysis, 20 µg of total protein was diluted in a loading buffer containing 2-mercaptoethanol and boiled for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, BIO-RAD), transferred to nitrocellulose, and stained with Ponceau S solution. The blots were blocked with 5% non-fat milk in PBS/0.05% Tween 20 for 1 h at RT and, subsequently, incubated for 24 h at 4°C with the primary antibodies listed in **Table 2** [↗](#). Lamin B1 (Proteintech) protein or bands detected after Ponceau S solution staining were used as a reference for normalization of the TTR monomer (Proteintech) or TTR tetramer (DSHB) levels in blood serum, respectively. For TTR monomer detection, the blots were probed with the respective HRP-conjugated secondary antibody (anti-rabbit or anti-mouse, 1:5000; Jackson ImmunoResearch, Suffolk, UK). The immunoreaction was detected using the ECL substrate (ThermoFisher Scientific, Waltham, MA, USA). For fluorescent Western blot analysis, a secondary antibody directed against mouse IgG, labeled with the Alexa Fluor 488 (Jackson ImmunoResearch; 1:1000) was used.

4.12. Statistics

Statistics was performed using GraphPad Prism 5 (version 5.00) and R v. 4.1.2 in RStudio. In GraphPad, statistical analyses were conducted for Western blots and some qPCR analyses using T-tests when comparing one variable. In R software, the analyses for qPCRs and organoid size were performed. Firstly, the Levene test was calculated to estimate variance. For equal variance, one-way ANOVA was used for multi-group analysis, followed by post-hoc T-tests (qPCR without p-value adjustment; organoid size with Bonferroni adjustment). In case the variance was not equal the, Welch's ANOVA was used, followed by post-hoc Welch's T-tests (qPCR without p-value adjustment; organoid size with Bonferroni adjustment).

4.13. Data availability

Data from single-cell sequencing of human fused brain organoids and mouse embryonic brain samples are stored on ZENOD in FASTQ (after QC) and normalized count matrix (after analysis) with metadata formats for organoids [ZENODO link] and embryonic brains [ZENODO link].

Antibody	Company name	Host	Diltution
TBR1	ProteinTech	Rabbit	1:400
PAX6	DSHB	Mouse	1:20
NOTCH1	DSHB	Rat	1:50
NEFH	DSHB	Mouse	1:50
NES	DSHB	Mouse	1:50
MAP2	DSHB	Mouse	1:50
GSH2	MERCK	Rabbit	1:500
FOXG1	Abcam	Rabbit	1:400
DLX2	ThermoFisher Scientific	Rabbit	1:100
NKX2.1	RD Systems	Rabbit	1:250
TTR	DSHB	Mouse	1:50
TTR	Proteintech	Rabbit	1:1000
SMI-32	BioLegend	Mouse	1:400
LAMIN B1	Proteintech	Mouse	1:2000

Table 2.

List of antibodies used in immunohistochemistry and/or western blot experiments.

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Additional information

The authors declare no competing or financial interests.

Author contributions

K.Ś-K. generated brain organoids and performed qPCR and immunofluorescence experiments. J.K. processed the embryonic tissue and organoids for scRNAseq, performed scRNAseq library synthesis, designed scRNAseq bioinformatic tools and analyzed the scRNA-seq data; M.F. and J.K. designed the scRNA-seq experiments and collected the embryonic tissue; J.D. and B.K. generated brain organoids, M.S., Ż.K-P and P.P. collected the serum and adult brains; L.H. J.P. M.R. A.S.-C. helped with RNAseq conduction; M.H. and N.C. provided resources, the HD mouse model; K.Ś-K., J.K. and M.F. wrote the article. M.F. was responsible for the concept and obtaining funding.

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Reviewer #1 (Public review):

In the manuscript "Identification of neurodevelopmental organization of the cell populations of Juvenile Huntington's disease using dorso-ventral HD organoids and HD mouse embryos," the authors establish a fused dorso-ventral system that mimics cortex-striatum interactions within a single organoid and use this system to investigate neurodevelopmental impairments caused by HD. Specifically, they describe certain phenotypes in 60-day HD organoids and the brains of humanized mouse embryos, utilizing both wet-lab and single-cell sequencing techniques. The authors also develop dorsal/ventral and ventral/dorsal mosaic control/HD organoids, showing a capacity to rescue some HD phenotypes.

The manuscript could be a valuable contribution to the field, however it has relevant drawbacks, the most significant being a lack of clarity regarding the replicates used for each genotype in the sequencing analyses. The lack of information on replicates raises the possibility that only a single replicate was analyzed for each organoid and brain sample. This approach may lead to concerns regarding the reproducibility of the findings, and it may be necessary for the authors to generate additional data to strengthen their conclusions. In addition, the analysis of the HD samples was conducted by pooling distinct cell populations from different brain regions (CTX, HIP, ChP for the dorsal brain, and STR, HYP, TH for the

ventral brain). It is unclear why scRNA seq was used on pooled brain regions, which could obscure region-specific insights.

Another issue pertains to their proposed outcome: "Finally, we found that TTR protein, a choroid plexus marker, is elevated in the adult HD mouse serum, indicating that TTR may be a promising marker for detecting HD". This statement appears to lack statistical support, which makes this set of data potentially misleading and inconclusive.

The authors are encouraged to provide evidence of biological replicates, remove outcomes that lack statistical support, and address a series of points as detailed elsewhere.

<https://doi.org/10.7554/eLife.102879.1.sa1>

Reviewer #2 (Public review):

The article titled "Identification of neurodevelopmental organization of the cell populations of juvenile Huntington's disease using dorso-ventral HD organoids and HD mouse embryos" analyses an in vitro human brain organoid model containing dorsal and ventral telencephalon structures derived from human iPSC from Huntington's disease patients or control subjects.

The authors describe differences in the pattern of expression of genes related to proliferation and neuronal maturation, with a slower pattern of differentiation present in HD cells. Moreover, the authors described a higher differentiation capacity of HD cells to generate choroid plexus identity following dorsal telencephalon prime protocol differentiation when compared to control cells. Whereas the claims related to Choroid plexus identity are intriguing, most of the claims made through the manuscript are not sustained by quantitative data or consistent results in the different conditions analysed, or many experiments seem to be missing to reach final conclusions.

In addition, the quality of the organoids used for experiments does not seem to have been assessed or satisfactorily presented in the figures of this paper. Many important details related to the experimental execution are missing in the current version of this manuscript.

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