

Utilization of Human Choroid Plexus Brain Organoids to Study Fluid Osmoregulation with Response to Osmotic Imbalance

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Abstract

Background

Syringomyelia (SM) is a fluid-associated disorder characterized by a fluid-filled cavity, known as a syrinx, inside the spinal cord caused by cerebrospinal fluid (CSF) displacement. The clinical standard for treating SM is surgery, as molecular signatures are not fully understood. Human choroid plexus (ChP) brain organoids harness the ability to produce CSF-like fluid pockets that could serve as an answer to understanding human SM pathophysiology as a lack of *in vitro* models exists to investigate mechanisms of human syrinx formation/expansion to unlock molecular understanding for syrinx shrinking.

Methods

Human ChP brain organoids were created from human induced pluripotent stem cells (hiPSCs) and the CSF-like fluid osmolality was characterized. Under different osmotic stress conditions – isotonic, hypertonic, and hypotonic – the regulation of betaine contributing factors, water and ion transporters was investigated in response to the osmotic stress. Immunohistochemistry (IHC), transcriptomics, and osmolality analysis were performed on all human ChP brain organoids.

Results

We demonstrated that human ChP brain organoids could be formed from hiPSCs and develop CSF-like fluid producing pockets, shown by IHC (TTR, CLIC6). The osmolality of the CSF-like fluid showed physiological relevance to human CSF, indicating this organoid system as a viable *in vitro* SM model. After osmotic stress was induced, IHC and transcriptomics showed upregulation of betaine contributing factors (BGT-1/SLC6A12, CHDH), water transporters (AQP1, AQP4), and ion transport (KCC4/SLC12A7) for hypertonic or hypotonic conditions compared to isotonic. Bulk RNA-sequencing transcriptomics indicated upregulation in pathways related to betaine, osmotic, transport, and CSF-like production for human ChP brain organoids in response to osmotic stress, a possible narrowing down of molecular etiology on how to manipulate the syrinx.

Conclusions

In summary, these results suggest the human brain ChP organoid system can be an *in vitro* model for studying SM and potential therapies as these results highlight key molecular players that could be targeted for non-surgical (*i.e.*, pharmaceutical) interventions for syrinx removal.

BACKGROUND

In the last two decades the biomedical/biomolecular research field has made tremendous progress in developing three-dimensional (3D) cell culture systems using stem cells [1–3] that have enabled new tools to explore the pathophysiology of various diseases, strategizing and testing personalized treatments, and drug screening methodologies [3–6]. With this progress, different kinds of spheroids and organoids culture approaches were developed. Spheroids are cell aggregates formed from two or more cell types that mimic a tissue or microtumor of interest, whereas organoids are self-assembled 3D tissue cultures derived from the stem cells that mimic a specific organ. Extensive efforts have been made by researchers to develop organoids that recapitulate different organs in the human body [4–6].

Cerebral organoids, also commonly known as brain organoids, have paved different paths for investigating neurodevelopment, neurological diseases, neurodegenerative disorders, mental illness, and neurotoxicity [2, 7, 8]. Brain organoids are lab-grown *in vitro* cell structures that resemble the early stages of embryonic brain development. Importantly they utilize human induced pluripotent stem cells (hiPSCs) or human embryonic stem cells (hESCs) that self-assemble and grow into 3D brain-like architectures [2]. These brain organoids develop characteristic central nervous system (CNS) cells including neurons, glia, and progenitor cells that recapitulate form and function of the human brain at the cellular, molecular, and tissue levels [9]. Anatomically, the adult human brain is divided into regions responsible for different functions, and extensive effort has been carried out to produce region-specific brain organoids to study region-specific aspects including during brain development and neurological disorders [10–15]. The recent development of cerebrospinal fluid (CSF)-like fluid-producing choroid plexus (ChP)-specific brain organoids is valuable due to the importance of the CSF and its movement in certain fluid-associated disorders in the CNS including but not limited to hydrocephalus, edema in the brain, and syringomyelia (SM) in the spinal cord [16].

The human ChP is a specialized secretory epithelial tissue located within the ventricular system of the brain. The main proposed function of the human ChP is to produce CSF in the CNS. It is comprised of epithelial cells on the basal lamina lining the ventricles that produce CSF to be transported throughout the CNS. CSF is utilized by the brain and spinal cord for mechanical support (cushioning), a medium for selected nutrient transport, disposing of undesirable by-products generated during metabolism, and hormonal signaling [17–19]. The single layer of epithelial cells is situated on a basement membrane and possesses well-grown microvilli. These microvilli provide increased surface area for choroidal epithelial cells for substantial secretion of CSF and other important required transport functions [18, 20, 21]. Choroidal epithelial cells are connected by the tight junctions that form the blood-CSF barrier (BCSFB). This barrier plays a vital role along with the blood-brain barrier (BBB) in regulating the CNS microenvironment for the proper functioning of the neurons and glia [22–26]. Since the human ChP is present deep inside the brain is poorly inaccessible to most analysis techniques, thus there exists a poor understanding of its microanatomy and utilization for biological research, and prior work has largely utilized animal models. To meet this need, Laura Pellegrini *et. al.* established the first human ChP brain organoid model that produces CSF-like fluid pockets integrated with cerebral type organoids [16].

Our previous research on syringomyelia (SM) proposed the potential involvement of betaine-focused fluid osmoregulation in SM pathophysiology utilizing a combination of *in vitro* and *in vivo* models [27, 28]. SM is characterized by a fluid-filled cavity, also known as the syrinx, inside the spinal cord caused by CSF displacement due to some combination of physical, biochemical, and osmotic disturbances [29–31]. Since SM is fluid associated disorder, in this study we were interested to determine if the human ChP brain organoid model could be used to study human fluid osmoregulation aspects under osmotic conditions akin to what is found in the injured CNS. Due to the lack of human 3D *in vitro* models for SM research, the objective was to develop a CSF-like fluid-producing ChP brain organoid model from human iPSCs and to attempt to study and link mechanisms of human syrinx formation/expansion and fluid osmoregulation-related responses. Herein, ChP brain organoids were developed from hiPSCs due to their ability to self-organize into 3D brain organoids given an established set of chemical and physical cues [32], then the CSF-like fluid osmolality was characterized. Next, regulation of betaine contributing factors including synthesis enzyme choline dehydrogenase (CHDH) and betaine-GABA transporter 1 (BGT-1) were studied in the choroidal epithelial layer under varied osmotic stress conditions – isotonic (iso), hypertonic (hyper), and hypotonic (hypo). Similarly, water and ion transporter regulation were also investigated in response to osmotic stress using this model. Transcriptomics from bulk RNA-sequencing showed the differential gene expression and pathways impacted by the osmotic stress. The preliminary results obtained in this study are relevant to SM fluid osmoregulation and showed the potential to utilize this model for further extensive investigation of understanding SM pathophysiology.

METHODS

Stem Cell Culture

hiPSCs (IMR90-4) obtained from WiCell (WI, USA) were used for making ChP brain organoids. These cells were cultured and maintained in mTeSR™ Plus Medium (STEMCell Technologies) in surface-treated cell culture flasks or 6 well-plates using 1% Matrigel (Corning) [2]. These cells were maintained in an incubator at 37°C under 5% CO₂/95% air and cultured, passaged, and used for the required experiments under the standard sterile conditions in a biosafety cabinet in a dedicated culture room.

Embryoid body formation from hiPSCs

To initiate embryoid body (EB) formation, following the protocol from Pellegrini, L. *et al* [16], hiPSCs were cultured in a round bottom low-attachment 96 well-plate (Corning) with a 2,500 cell/well initial density and were maintained in embryonic body medium (EBM). EBM consisted of DMEM-F12 (Gibco, Thermo Fisher Scientific), Knockout serum replacement medium (KOSR) (Gibco, Thermo Fisher Scientific), fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), Glutamax (Gibco, Thermo Fisher Scientific), MEM-NEAA (Gibco, Thermo Fisher Scientific), 2-mercaptoethanol (Acros, Thermo Fisher Scientific), and penicillin-streptomycin (Gibco, Thermo Fisher Scientific). The EBM was replenished every 48 hours for 6 days. After 6 days, EBs were transferred from round bottom low-attachment 96 well plate to intermediate

induction medium (IIM) in low-attachment flat-bottom 24 well plates. The IIM consisted of DMEM-F12, KOSR, FBS, N2 supplement, Glutamax, MEM-NEAA, penicillin-streptomycin, and heparin (Millipore-Sigma). The next day, EBs were replenished with a neural induction medium (NIM), the same composition as IIM without KOSR and FBS.

ChP brain organoid development

This is a continuation of the EB formation process above and followed the protocol from [16]. On day 8, EBs were embedded in a 35µl droplet of Matrigel® using sheets of dimpled and sterilized parafilm. It was conducted in such a way that EB was surrounded by Matrigel®. Embedded EBs were incubated at 37°C for 20 min in 162 incubators. Embedded EBs were transferred to a 6-well plate with 4 ml of cerebral differentiation medium (CDM) without vitamin A. CDM comprises DMEM-F12, neural basal media, N2 supplement, Glutamax, MEM-NEAA, penicillin streptomycin and 2-mercaptoethanol, B27 without vitamin A supplement (Gibco, Thermo Fisher Scientific). On day 10, 3 µM CHIR (Millipore=Sigma) and 20 ng/ml bone morphogenetic protein 4 (BMP4) (Peprotech, Thermo Fisher Scientific) with CDM were fed to EBs for ChP patterning. This treatment was kept for 7 days. After this CDM with vitamin A (CDM + A), media was changed every 6 days. ChP organoids were agitated on an orbital shaker (57 rpm) from day 18 onwards to encourage nutrient and waste diffusion. Matrigel (1:50) was dissolved in CDM + A media from day 30 onwards until end points.

Fluid harvesting from fluid-filled pockets of ChP brain organoids

Fluid was harvested from the fluid-filled pocket of ChP brain organoids using a microneedle on day 60 for osmolality analysis. Before harvesting fluid, media was kept as minimal as possible in the well to avoid contaminating fluid from the media while harvesting it from the pocket. Fresh media and D60 media were concurrently tested to analyze osmolality. The osmolality of collected pocket fluid and media fluids were analyzed with a vapor pressure osmometer (Vapro 5600, Wescor Inc.) [33] to determine the total osmolality of each solution. D60 pocket media and D60 media osmolality were statically analyzed compared to fresh media.

Solute/water/ion transporter/channel and related enzyme expression in response to osmotic stress

Three different osmotic conditions – isotonic, hypotonic, and hypertonic – were used to study fluid osmoregulation in ChP brain organoids. The expression of targeted channels/transporters/enzymes was studied in the ChP epithelial layer around CSF-like fluid-filled pockets. The expression of solute channels (BGT-1), synthesis enzyme (CHDH), water channels (AQP1 and AQP4), and ion channels (KCC4) were evaluated in response to the osmotic conditions. ChP brain organoids were exposed to different osmotic conditions (iso: media, hyper: 0.5% NaCl, hypo: 1:1 media:water) overnight, then corresponding

organoids were fixed, embedded, sectioned, stained, and imaged for the target channels/transporters/enzymes.

Immunostaining (IHC)

After washing in warmed PBS, ChP brain organoids were fixed in 4% paraformaldehyde (PFA) for 45 minutes. The fixed organoids were next embedded in optimal cutting temperature (OCT) (Tissue-Tek) medium and stored at -20°C. The OCT-embedded ChP brain organoids were then sectioned using a cryostat microtome (Leica CM 1850, Wetzlar, Germany) with 15 µm thickness and stored at -20°C until further usage. These sections on slides were baked for 30 min in an incubator at 37°C before starting the staining procedure. Sections were permeabilized with 0.25% of Triton X and were blocked by 10% FBS in phosphate buffered saline (PBS). The sections were incubated with primary antibodies for 1 hr at room temperature. After 3 washes with PBS, sections were stained with secondary antibodies for an hour at room temperature, the whole procedure was conducted in the dark after the addition of the secondary antibody. In the case of co-staining (for more than two targets), sections were incubated with primary and secondary antibodies for the second target in the same manner as explained. The washing in between staining was conducted with 1x PBS. The cell nuclei were stained with 10 µM of DAPI solution for 7 minutes at room temperature in the dark. After washing with 1x PBS, sections were mounted with Prolong Gold using coverslips, and then it was air-dried overnight. The slides were moved to 4°C until further imaging. For ChP confirmation, two ChP specific markers transthyretin (TTR) (polyclonal, Thermo Fisher Scientific), and chloride intracellular channel protein 6 (CLIC6) (polyclonal, Thermo Fisher Scientific) were used. Targets in relation to osmotic stress, BGT-1 (Anti-BGT1 (SLC6A12) Antibody, Alomone Laboratories), CHDH (sc-393885, Santa Cruz Biotechnology), AQP1 (sc-25287, Santa Cruz Biotechnology), AQP4 (sc-32739, Santa Cruz Biotechnology), and KCC4 (PA5-59666, Thermo Fisher Scientific). Images were obtained using a Nikon A1 confocal microscope and processed using ImageJ software (NIH).

Transcriptomics

Transcriptomics was used to determine the overall gene expression after osmotic stress was applied to the ChP brain organoids. ChP brain organoids (n = 8/condition) were stored in RNA/later Stabilization Solution (Invitrogen, Thermo Fisher Scientific) until RNA extraction. Total RNA was extracted from homogenized samples using the RNeasy Lipid Tissue Mini Kit for High-Yield RNA (Qiagen) according to the manufacturer's protocol and quantified on a Qubit. Samples for each osmotic condition were pooled for technical replicates, n = 4/condition. RNA-seq libraries were prepared using the NEBNext Ultra II RNA kit and sequenced on an Illumina NovaSeq X (100 bp) at the Case Western Reserve University (CWRU) Genomics Core Facility.

RNA-seq data analysis

RNA-seq analysis was performed using human reference annotations. A total of 12 paired-end FASTQ files (four biological replicates per condition) were generated with an initial read length of 150 bp. Prior to alignment, raw reads were quality-trimmed to 75 bp to remove low-quality bases and improve mapping accuracy. Trimmed reads were aligned to the human reference genome and quantified using RNA-seq by Expectation Maximization (RSEM), which simultaneously performs read alignment and transcript abundance estimation. Gene-level quantification was conducted, including expected read counts and Transcripts Per Million (TPM) values.

Differential gene expression (DEG) analysis was performed by comparing each treatment condition against the control using gene-level expected counts obtained from RSEM. Statistical testing was conducted across biological replicates to identify genes with significant expression changes between conditions. The criteria used for performing the DEGs are False Discovery Rate (FDR) < 5% and |Fold Change| > 2. This filtering strategy ensured that identified DEGs were both statistically significant and biologically meaningful.

To investigate the biological significance of the differentially expressed genes, pathway enrichment analysis was performed separately for each treatment-control comparison using ReactomePA. The enrichment p-values were further adjusted by Benjamini–Hochberg (BH) multiple test correction.

Statistical analysis

Osmolality and gene fold change results were analyzed via one-way ANOVA ($\alpha = 0.05$) with Tukey's *post-hoc* using Prism 9.0 software (GraphPad).

RESULTS

Development of ChP brain organoids

Utilizing ChP-specific cues, the Lancaster lab modified established cerebral organoid protocols to formulate an *in vitro* human ChP model that accurately recapitulated physiological *in vivo* functions [16]. We utilized this protocol to implement the ChP brain organoid model (Fig. 1A). Figure 1B depicts the schematic of the timeline to form EBs from hiPSCs and ChP organoid from EBs, and the details including required media. Representative images of important steps in the *in vitro* culture process are shown in Fig. 1C, with more detailed images of the stepwise process in Supplemental Fig. 1. The matured ChP brain organoids with CSF-like fluid pockets at day 55 (Fig. 1C) were subjected to three osmotic conditions – isotonic, hypertonic, and hypotonic – overnight before being used for subsequent transcriptomic analysis (Fig. 1D). ChP brain organoids with large cystic formation had visible reactions to osmotic conditions, with hypertonic causing puckering, and hypotonic causing swelling (Fig. 1E, Supplemental Fig. 2A). The ChP brain organoids with larger cystic structures sometimes form and allow the CSF-like fluid to be removed for further osmolality analysis (Supplemental Fig. 2B).

Bulk RNA-sequencing of ChP brain organoids

Transcriptomic analysis of ChP brain organoids after osmotic stress conditions was performed to further analyze how environmental stress impacted gene expression, with all analysis comparing hypertonic or hypotonic to isotonic (control) conditions. Following RSEM analysis, the Principal Component Analysis (PCA) plot revealed tight, independent grouping of all ChP brain organoids per osmotic condition (Fig. 2A). The top 20 enriched pathways across the osmotic treatment conditions showed an overall increase for the hypertonic condition, conversely the hypotonic condition showed a decrease in the same pathways (Fig. 2B). When specifically looking at pathways that influence betaine, osmotic, transport, and CSF production, most pathway activity increased for the hypertonic ChP organoids, while hypotonic treatment showed decreased pathway activity (Fig. 2C). Interestingly, the solute carrier (SLC)-mediated transmembrane transport, aquaporin-mediated transport, transport of small molecules, and SLC-mediated transport of neurotransmitters pathways showed increased activity for the hypertonic condition, with hypotonic showing decreased pathway activity (Fig. 2C). Total DEGs, 351 and 3349 for hypertonic and hypotonic, respectively, revealed the hypotonic condition had a larger impact on gene expression (Fig. 2D-E), with the full DEG breakdown shown in Supplemental Fig. 3. Both groups had more DEG upregulated than downregulated (Fig. 2D-E). Specific regulations for genes that are a part of ChP brain organoid characterization (TTR, CLIC6), CSF markers (NFAT5, AKR1B1, SLC5A3, SLC14A1, SLC6A6, HSPA2, SLC38A2), osmolality (CLU, IGF2), betaine pathways (BGT-1/SLC6A12, CHDH), water transport (AQP1, AQP4), and ion transport (KCC4/SLC12A7), are explored in subsequent results sections.

Characterization of ChP brain organoids

The development of ChP brain organoids was primarily confirmed using IHC of sectioned organoids using the choroidal epithelial-specific markers TTR and CLIC6. A high level of expression of both TTR and CLIC6 was observed in fluid pockets as well as in ChP brain organoids, indicating commitment towards ChP specific cells (Fig. 3A). Fold change analysis, comparing hypertonic and hypotonic to isotonic, for TTR and CLIC6 expression indicated no significance and hypotonic upregulation, respectively, indicating that the osmotic conditions did not fundamentally change the ChP brain organoids (Fig. 3B). A $\log_2$ fold change (FC) analysis showed no change in TTR regulation, while CLIC6 was upregulated for the hypotonic condition (Fig. 3C).

Osmolality of CSF-like fluid from ChP organoid pockets

Further confirmation of ChP brain organoid development was conducted by analyzing the osmolality of fluid from the fluid pocket of the ChP organoid (Fig. 4A). Here, we found that the average osmolality of the pocket fluid is 311 ± 23 mmol/kg at day 60 ChP brain organoid (Fig. 4B). When compared to fresh media (307 ± 4 mmol/kg), D60 media osmolality (353 ± 29 mmol/kg) was statistically different ($p = 0.0498$), while the D60 pocket fluid osmolality was not significantly different ($p = 0.9630$) (Fig. 4B). CSF markers, CLU and IGF2, were also investigated to further ensure the fluid produced by the ChP brain

organoids was biochemically similar to CSF. CLU was upregulated for both hypertonic and hypotonic conditions, with the latter having the most expression (Fig. 4C-D). IGF2 expression was similar across all conditions, as no significant up or down regulation occurred (Fig. 4C-D). Key osmotic markers such as NFAT5, AKR1B1, SLC5A3, SLC14A1, SLC6A6, HSPA2, and SLC38A2, were evaluated for expression of hypertonic and hypotonic compared to isotonic (Fig. 4E). Looking at hypertonic, NFAT5, SLC5A3, SLC6A6, HSPA2, and SLC38A2, were upregulated, while AKR1B1 and SLC14A1 were downregulated (Fig. 4E-F). The opposite was true for hypotonic, as NFAT5, AKR1B1, SLC5A3, SLC14A1, SLC6A6, and SLC38A2 were downregulated, and HSPA2 was upregulated (Fig. 4E-F).

Betaine contributing factors in choroidal epithelial cells with response to osmotic stress

Since there is no preferred living human 3D *in vitro* model for SM, we sought to study the feasibility to utilize this CSF-like fluid-producing ChP organoid model due to the closely relevant nature of the disorder by exposing the system to three different osmotic conditions – isotonic (control), hypertonic, and hypotonic solutions. Based on our previous animal work [27, 28], BGT-1 and CHDH were isolated as key SM markers, and they were shown to regulate local cellular volume and osmoregulation during osmotic challenge, highlighting the importance of studying BGT-1 and CHDH expression in ChP brain organoids (Fig. 5). Starting with IHC, TTR was used as a control marker, since ChP organoids should remain fundamentally unchanged even after osmotic condition exposure. After being exposed to hypertonic insult, BGT-1 expression was seen to slightly increase and localized in an epithelial layer, however, no BGT-1 expression was visible when ChP brain organoids were exposed to isotonic and hypotonic conditions for IHC analyses (Fig. 5A). Looking at BGT-1 mRNA expression fold change and $\log_2FC$ results, both hypertonic and hypotonic challenge increased expression compared to isotonic controls (Fig. 5C-D). A similar observation was found with CHDH expression that increased expression was found in only hypertonic conditions as compared to isotonic and hypotonic conditions as shown by IHC (Fig. 5B). CHDH gene expression, via fold change and $\log_2FC$, showed slight downregulation for hypertonic and a slight upregulation for hypotonic conditions (Fig. 5C-D).

Next, we moved to the study of aquaporin water channels (AQP1 and AQP4) and ion transport channel (KCC4) because these channels are established to control the macroscopic movement of water and ions across the BBB/BCSFBB [22–26]. We showed here via IHC robust expression of AQP1 in all osmotic conditions but relatively higher expression of AQP1 in hypertonic conditions were observed as compared to isotonic and hypotonic conditions (Fig. 6A). Unlike AQP1, we found no expression of AQP4 in isotonic and hypotonic conditions but found increased expression in hypertonic stress conditions via IHC (Fig. 6B). The expression of KCC4 in choroidal epithelial cells was similar across all osmotic conditions from IHC (Fig. 6C). Gene expression from fold change and $\log_2FC$ analysis of AQP1, AQP4, and KCC4 showed no significant up or

downregulation for all treatment groups (Fig. 6D-E).

DISCUSSION

Previous SM research by our lab has highlighted a lack of understanding of the SM syrinx and development of human *in vitro* models able to more accurately depict SM pathophysiology that can be harnessed for researching future therapies. Organoid systems have become a valuable model source for studying complex neurological diseases, such as SM [2, 7, 8]. Thus, we aimed to utilize the ChP brain organoid model developed by the Lancaster lab, as these organoids grow cystic structures that produce a CSF-like fluid, similar to what is found in the CNS (Fig. 1A-B) [16]. ChP brain organoids are differentiated from other brain organoids utilizing two main signaling components – BMP4 (dorsalizing factor) and CHIR (Wnt-activator). These two small molecules are added at ~ day 10 post EB formation to provide the needed cues for the derivation of ChP cells (Fig. 1B). These factors were previously reported to promote choroidal epithelial cells in 3D *in vitro* culture [34, 35] and hence were applied in this protocol for promoting partial ChP fate decisions in cerebral organoids (Fig. 1A-B) [16]. In this study, ChP organoids were developed from hiPSCs within 15 days and matured until D55, before being subjected to three osmotic conditions – isotonic, hypertonic, and hypotonic (Fig. 1C-E) – with the hypertonic condition expected to mimic the SM environment *in vivo* [27, 28].

To better understand molecular responses to osmotic stress, we analyzed ChP brain organoids challenged in varying osmotic conditions using bulk RNA-seq to map global transcriptomic changes and pathway activity. PCA plotting, following RSEM analysis, indicated that for each osmotic treatment group, tight expression occurred, meaning ChP brain organoids that underwent each osmotic treatment had similar gene expression profiles (Fig. 2A). The hypertonic condition upregulated the top 20 enriched pathways, but these pathways shared few evident biological connections with regards to modeling SM pathophysiology (Fig. 2B). The top 20 selected pathways that related to betaine, osmotic, transport, and CSF processes were more active in the hypertonic condition, with the hypotonic condition showing less pathway activity (Fig. 2C). While hypertonic environments trigger a less immediate critical cell shrinkage, hypotonic conditions produce far more severe consequences, often culminating in cell rupture, lysis, and cell necrosis [36]. As a result, due to less immediate impacts on the cell, hypertonic environments have less complex transport activity pathways active, as the main regulation mechanism is passive water transport out of the cell [36]. Overall, no active transport is needed, and the simplified regulation allows hypertonic pathways to be more easily activated, and thus upregulated, compared to cells exposed to hypotonic environments [36]. This phenomenon is further exemplified when looking at the total DEGs (Fig. 2D-E). ChP brain organoids in the hypertonic environment had fewer total DEGs, 351, compared to the hypotonic total DEGs, 3349 (Fig. 2D-E). Transcriptomic analysis proved that in terms of using the ChP brain organoids as a human *in vitro* model for SM, the hypertonic environment caused expression similar to what has been seen previously in animal SM modeling [27, 28].

As previously mentioned, a defining feature of ChP organoids is the ability to form colorless CSF-like filled fluid pockets and the cell-based layer holding these pockets roughly confirms *in vitro* choroidal plexus development (Fig. 1C) [16]. The TTR and CLIC6 positive choroidal epithelial layers are important for maintaining a separate and intact fluid pocket (Fig. 3A). Further, the fluid-producing capacity of these

organoids and choroidal epithelial markers confirmed the production of ChP brain organoids (Fig. 3A). These results align with the original work reporting the creation of ChP brain organoids [16]. Also, introducing these ChP brain organoids into different osmotic environments did not fundamentally change TTR gene expression (Fig. 3B-C). Upregulation of CLIC6 for hypotonic conditions occurred but should not have had detrimental impacts on the model, as this channel is known to be sensitive to cell volume changes (Fig. 3B-C) [37]. Overall, the established ChP brain organoid protocol produced organoids that were relevant to achieving a 3D *in vitro* model for SM.

Clinically, the concentration of active solutes in biofluids is evaluated through osmolality analysis – a diagnostic staple used to screen for sodium balance disorders, including hyponatremia and hypernatremia [38, 39]. Therefore, osmolality of the syrnix fluid was investigated to confirm the clinical relevance of the ChP brain organoid model. The confirmation of fluid produced by these brain organoids was previously confirmed in the original study via protein profile, expression of transporter AQP1, and key enzymes for generation of the gradient driving CSF production [16]. The CSF-like fluid from the ChP organoids closely resembled *in vivo* CSF when their protein profiles were compared [16]. The measured average pocket fluid osmolality (311 ± 23 mmol/kg) from D60 ChP brain organoids further confirms the relevance to *in vivo* CSF osmolality (280–300 mmol/kg) (Fig. 4B) [40, 41]. Furthermore, the D60 media (353 ± 29 mmol/kg) showed a higher osmolality from fresh media (307 ± 4 mmol/kg) and the organoid pockets, suggesting that the organoids are able to establish an osmotic gradient. CSF-like production was investigated via the expression levels of CLU and IGF2, two key genes for CSF production [42–44]. CLU expression was upregulated in both hypertonic and hypotonic conditions, indicating CSF-like fluid production was influenced by the environment, which would be expected to combat drastic changes to homeostasis (Fig. 4C-D) [42–44]. IGF2, a growth factor playing a critical role in development and cell proliferation, did not show notable gene expression changes, indicating the osmotic conditions had no detrimental impacts on the ChP brain organoids (Fig. 4C-D) [42–44]. Exposure to varying osmotic environments triggered expected gene expression changes as the ChP brain organoids worked to maintain homeostasis. Crucially, the osmolality of the secreted CSF-like fluid remained without physiological limits (Fig. 4B), validating this system as a viable model for SM.

The critical roles of core osmotic markers such as NFAT5, AKR1B1, SLC5A3, SLC14A1, SLC6A6, HSPA2, and SLC38A2 in maintaining water balance, ion concentration, and macromolecular stability suggest that their expression profiles should be impacted in response to osmotic stress [43, 45]. In alignment with this homeostatic framework, our treatment groups in the ChP brain organoids demonstrated distinct transcriptional alterations across these key targets. The hypertonic treatment caused increased expression of the core osmotic markers when compared to isotonic control conditions (Fig. 4E-F) [43]. Both AKR1B1 and SLC14A1 were downregulated for hypertonic treatment, indicating cellular metabolism and water transport was decreased for these ChP brain organoids (Fig. 4E-F) [43]. Genes that are upregulated in hypertonic environments should be downregulated in hypotonic conditions, which was seen for most of the key osmotic genes (Fig. 4E-F). The only anomaly was HSPA2, which was upregulated in the hypotonic environment, indicating that the cellular response to osmotic stress likely requires higher expression of HSPA2 to combat hypotonicity (Fig. 4E-F) [43]. Hence, when exposed to

varying osmotic conditions overnight, ChP brain organoids adjusted during this short time period to maintain homeostasis, with some of these genes being potential targets to shrink the cystic structure.

The role of betaine focused fluid osmoregulation in SM has been extensively investigated to reveal the probable mechanisms to form and expand the syrinx in the spinal cord post-SCI [27, 28, 46, 47]. To follow up on this established work, in this study we investigated the expression of betaine contributing factors, transporter BGT-1, and a synthesis enzyme CHDH in the epithelial layer of the fluid-holding pocket of ChP brain organoids when exposed to the different osmotic stress conditions that relate to the SM environment. The hypertonic condition increased BGT-1 expression, while hypotonic and isotonic conditions had little to no impact on expression in CHP organoids (Fig. 5A). These results suggest that hypertonic stress upregulates the BGT-1 channel to selectively transport the potent osmolyte betaine in an attempt to actively balance the osmolality inside the CSF pocket and in the media. Similarly, CHDH was shown to be upregulated in hypertonic conditions as compared to the other two osmotic conditions (Fig. 5B). These results were expected, as CHDH synthesizes the osmolyte betaine, which can be subsequently transported by BGT-1 bi-directionally[48]; thus, a concurrent increase in both factors is consistent with a coordinated osmotic response. Fold change gene expression data showed increases in BGT-1 expression in both hypertonic and hypotonic solutions, contrary to IHC, but no statistical difference between hyper and hypo (Fig. 5C-D). CHDH expression was found to be upregulated in hypotonic but downregulated in hypertonic, again contradicting IHC results (Fig. 5C-D). The differences in gene vs protein expression could be due to IHC showing where BGT-1 and CHDH are located within cells, while global transcriptomics determines the amount of gene expression present in the entire organoid. The transcellular solute transport in the choroidal epithelial layer is reported in the literature and to a certain extent this solute transport affects CSF secretion [18], however, there was no mention of osmolyte molecules including betaine. However, the slight upregulation of both BGT-1 and CHDH seen in hypertonic conditions in epithelial cells via IHC is an interesting finding to relate this model with SM pathophysiology.

Specifically, characterizing AQP1 expression was critical to our study, given its predominant localization in the ChP and its documented role in mediating transepithelial water movement [18, 19]. IHC showed robust expression of AQP1 for ChP brain organoids in hypertonic conditions (Fig. 6A). It has been previously shown that the water permeability through AQP1 is linked to CSF production in the ChP [18]. Contrasting AQP1, AQP4 expression from IHC was minute across all three conditions (Fig. 6B). At the gene expression level, both AQP1 and AQP4 were not statistically different from isotonic controls (Fig. 6D-E), indicating active water transport was occurring but not at a level showing significance, but this does not completely rule out water transport as an option for modulating the syrinx in the more physiologically complex *in vivo* environment. If anything, the IHC results indicate localization of key transport channels, while the transcriptomic data reveals whether modulating expression fundamentally impacts syrinx architecture. In our previous SM molecular research [27, 28], we have speculated the potential involvement of AQP1 and AQP4 in water movement with response to osmotic imbalance or disturbance via BGT-1 and CHDH. The findings from this study showed relevance in terms of potential targets of SM, which could be further investigated using ChP brain organoids in future work.

Given that the potassium-chloride cotransporter KCC4 had been shown to be pathologically upregulated in the SM environment *in vivo* [27], characterization of this specific ion channel was critical to validating our model's ionic regulatory capacity. Hence, the expression of KCC4 in choroidal epithelial was studied via IHC analyses, showing that hypertonicity increased the expression of KCC4, likely to pump potassium and chloride ions through the epithelial layer of ChP brain organoid as compared to isotonic and hypotonic stress conditions (Fig. 6C). Gene expression once again showed no differences between the three osmotic conditions, but both hypertonic and hypotonic conditions resulted in higher expression than isotonic controls (Fig. 6D-E). In the literature, the expression of KCC4 was previously reported in ChP epithelial cells [18], which supports our findings in this study. Overall, the information gained from utilizing ChP brain organoids as an *in vitro* model of SM, highlights the lack of understanding for this disease and possible transport channels, molecules, and synthesis enzymes that could be targeted as a tactic to modulate the syrinx.

CONCLUSIONS

In summary, we confirmed the development of ChP brain organoids from hiPSCs using ChP specific markers and set about investigating their potential for studying CNS osmoregulatory functions. The osmolality of the fluid harvested from the pockets was found to be similar to human CSF osmolality, indicating physiological relevance. Hence, we utilized this model to further investigate betaine-focused fluid osmoregulation mechanisms in SM pathophysiology. We demonstrated the protein level upregulation of betaine regulating transporter (BGT-1) and synthesis enzyme (CHDH) along with water channels (AQP1 and AQP4), and ion transporter (KCC4) when ChP organoids were exposed to hypertonic conditions. The expression of these proteins in a choroidal epithelial layer under osmotic stress conditions showed relevance to our previous *in vitro* and *in vivo* studies where we showed the potential involvement of these targets in SM. The results from this preliminary study suggest potential for this model to be utilized in SM research due to its close relevance and human cell-based approach. While these preliminary findings underscore the robust potential of this system, extensive future investigations, including ChP brain organoids derived from other human iPSC or patient-derived cell lines, are essential to fully establish the model's utility in SM research and accelerate its transition toward personalized therapeutic applications.

Abbreviations

3D

three-dimensional

AKR1B1

Aldo-Keto Reductase Family 1 Member B1

AQP1

aquaporin 1

AQP4

aquaporin 4

BBB
blood-brain barrier
BCSFB
blood-CSF barrier
BGT-1
betaine-GABA transporter 1
BH
Benjamini-Hochberg
BMP4
bone morphogenic protein 4
CDM
cerebral induction medium
CDM + A
cerebral induction medium + vitamin A
CHDH
choline dehydrogenase
ChP
choroid plexus
CLIC6
chloride intracellular channel 6
CLU
Clusterin
CNS
central nervous system
CSF
cerebrospinal fluid
CWRU
Case Western Reserve University
DEG
differentially expressed genes
EB
embryoid body
EBM
embryonic body medium
FBS
fetal bovine serum
FC
fold change
FDR
False Discovery Rate

hESC
human embryonic stem cell
hiPSC
human induced pluripotent stem cells
HSPA2
Heat Shock Protein Family A Member 2
IGF2
Insulin-like Growth Factor 2
IHC
immunohistochemistry
IIM
intermediate induction medium
KCC4
potassium-chloride cotransporter 4
KOSR
knockout serum replacement
NFAT5
nuclear factor of activated T-cells 5
NIM
neural induction medium
n.s.
not significant
OCT
optimal cutting temperature
PBS
phosphate buffered saline
PCA
Principal Component Analysis
PFA
paraformaldehyde
rpm
revolutions per minute
RSEM
RNA-seq by Expectation Maximization
SLC
Solute Carrier
SLC12A7
solute carrier family 12 member 7
SLC14A1
Solute Carrier Family 14 Member 1

SLC38A2
Solute Carrier Family 38 Member 2
SLC5A3
Solute Carrier Family 5 Member 3
SLC6A12
solute carrier 6 family member 12
SLC6A6
Solute Carrier Family 6 Member 6
SM
syringomyelia
TPM
Transcripts Per Million
TTR
transthyretin

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare no competing interests.

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Authors' Contributions

DDP, KJB, and NDL planned experimental designs. DDP conducted *in vitro* studies, IHC, image acquisitions, image analysis, osmolality analysis, data analysis, and contributed to manuscript writing. KJB conducted *in vitro* studies, image acquisitions, image analysis, total RNA extraction, data analysis, and prepared the manuscript. GBS conducted RNA-seq, DEGs, and pathway enrichment analysis. KJB, DDP, GBS, JP, and NDL revised the manuscript and participated in data discussion and data interpretation. KJB and NDL participated in the production of the final manuscript.

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Figures

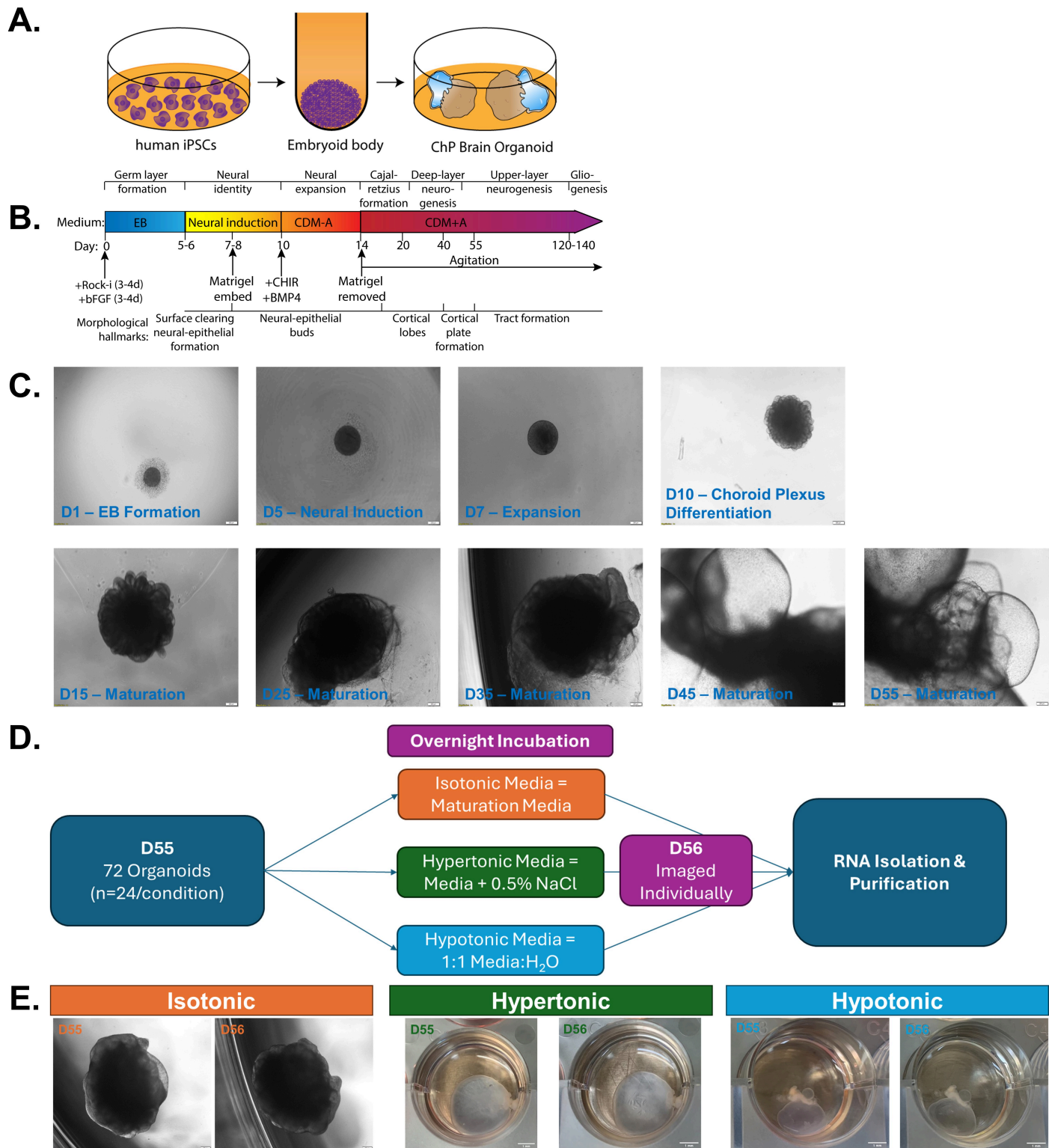


Figure 1

ChP brain organoid creation as an in vitro model of syringomyelia. (A) Schematic representation of EBs and ChP brain organoids formation from hiPSCs. (B) Timeline of ChP brain organoid generation with media and morphological hallmarks (modified from [16]). (C) ChP brain organoid generation at key morphological hallmark periods. Scale bar = 200 μ m. (D) In vitro experimental overview for ChP brain

organoids, n = 24. (E) Changes in D55 ChP brain organoid cystic formation after exposure to isotonic, hypertonic, and hypotonic conditions in vitro. Scale bars = 200 µm in C. and 1 mm in E.

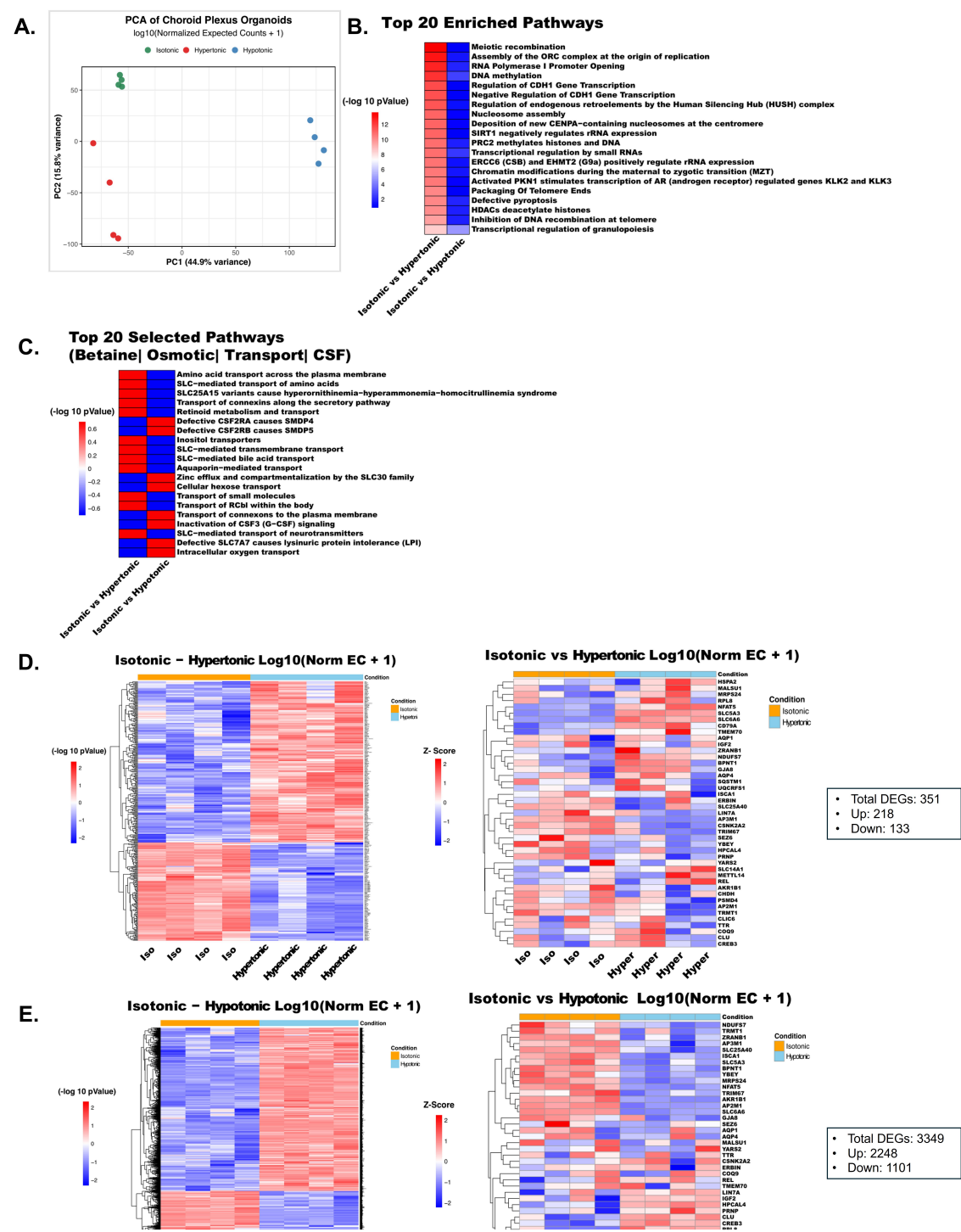


Figure 2

ChP brain organoid transcriptomics from bulk RNA-sequencing after RSEM analysis. (A) PCA plot. (B) Top 20 enriched pathways across treatment conditions. (C) Top 20 selected pathways for betaine,

osmotic, transport, and CSF production for the 3 different treatment groups. (D – E) Overall differential and specific gene expression of ChP brain organoids in hypertonic (D) and hypotonic (E) groups. Hypertonic and hypotonic treatments compared to isotonic, n = 4.

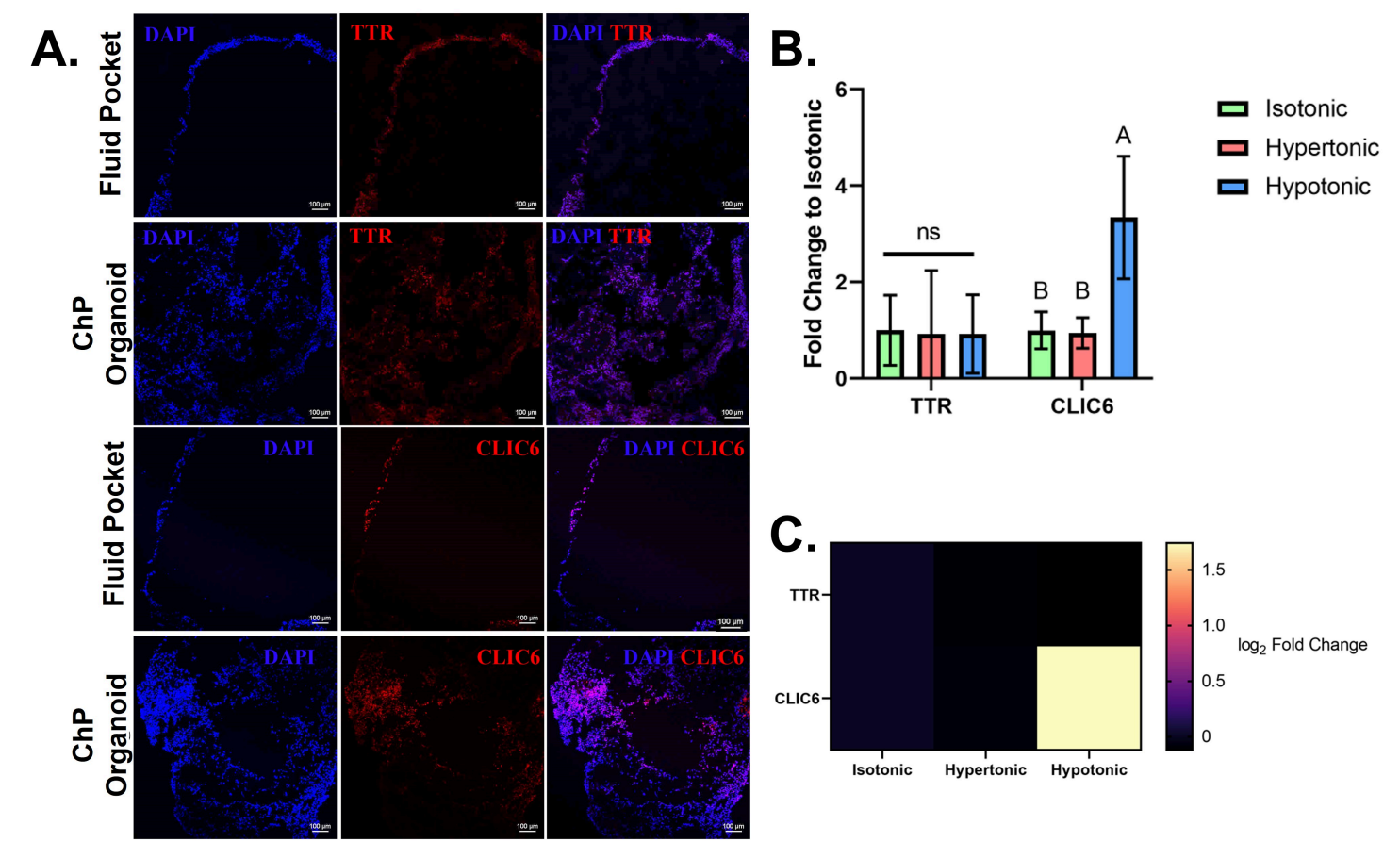


Figure 3

Confirmation of ChP brain organoid development. (A) Representative confocal images of sectioned day 45 ChP brain organoids stained for ChP specific markers, TTR and CLIC6 (both in red), with DAPI co-staining (blue). Scale bar = 100 μ m. (B – C) Fold change (B) and log₂ fold change heat map (C) of ChP specific genes after hypertonic and hypotonic treatment. (B: n = 4, 1-Way ANOVA with Tukey's *post-hoc*, TTR: p = n.s. = 0.9921, CLIC6: p = ** = 0.0029, treatments compared to isotonic; all data mean $\pm$ S.D.).

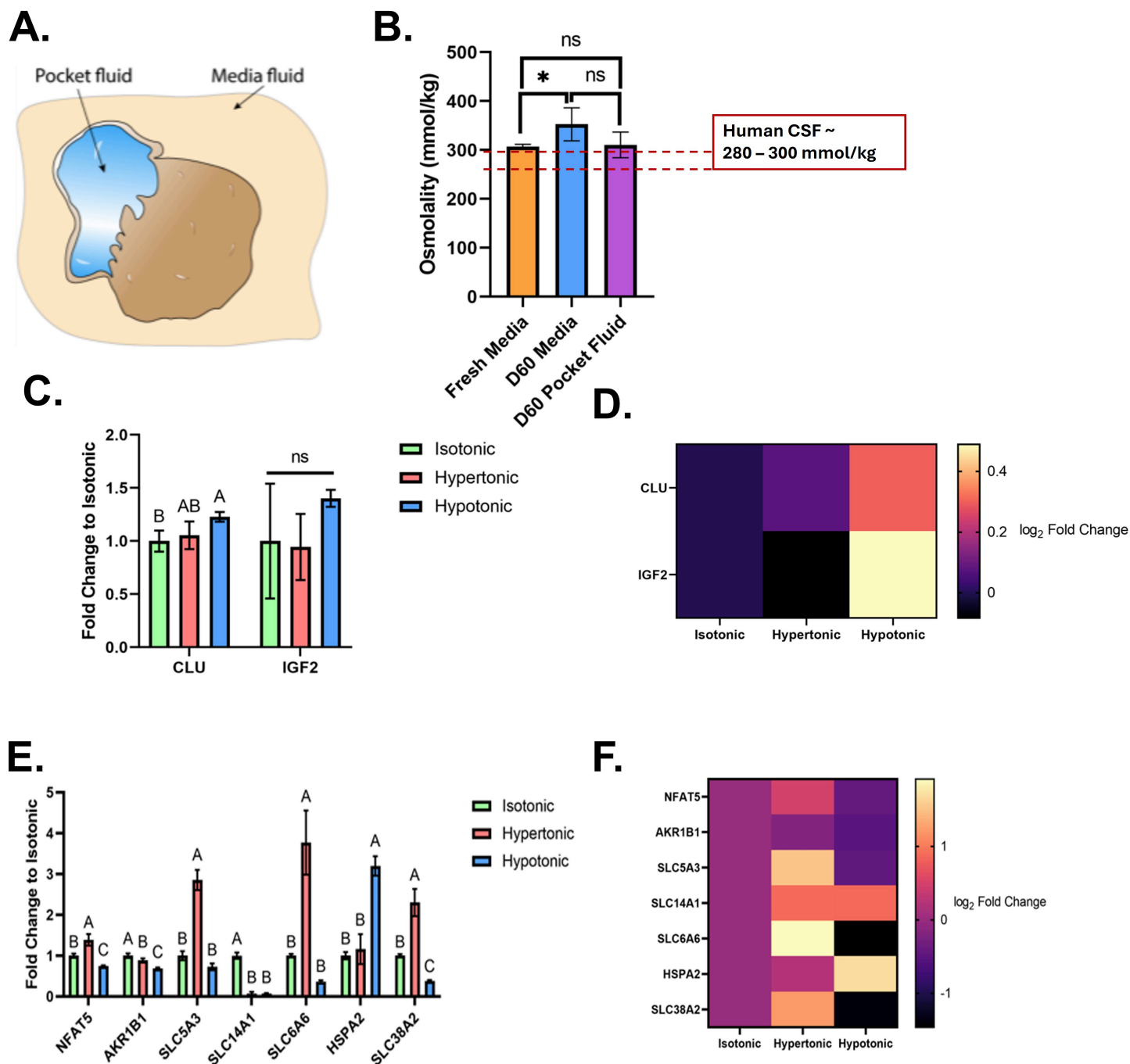


Figure 4

Confirmation of ChP brain organoid development. (A) Representative confocal images of sectioned day 45 ChP brain organoids stained for ChP specific markers, TTR and CLIC6 (both in red), with DAPI co-staining (blue). Scale bar = 100 μ m. (B – C) Fold change (B) and $\log_2$ fold change heat map (C) of ChP specific genes after hypertonic and hypotonic treatment. (B: n = 4, 1-Way ANOVA with Tukey's *post-hoc*, TTR: p = n.s. = 0.9921, CLIC6: p = ** = 0.0029, treatments compared to isotonic; all data mean $\pm$ S.D.).

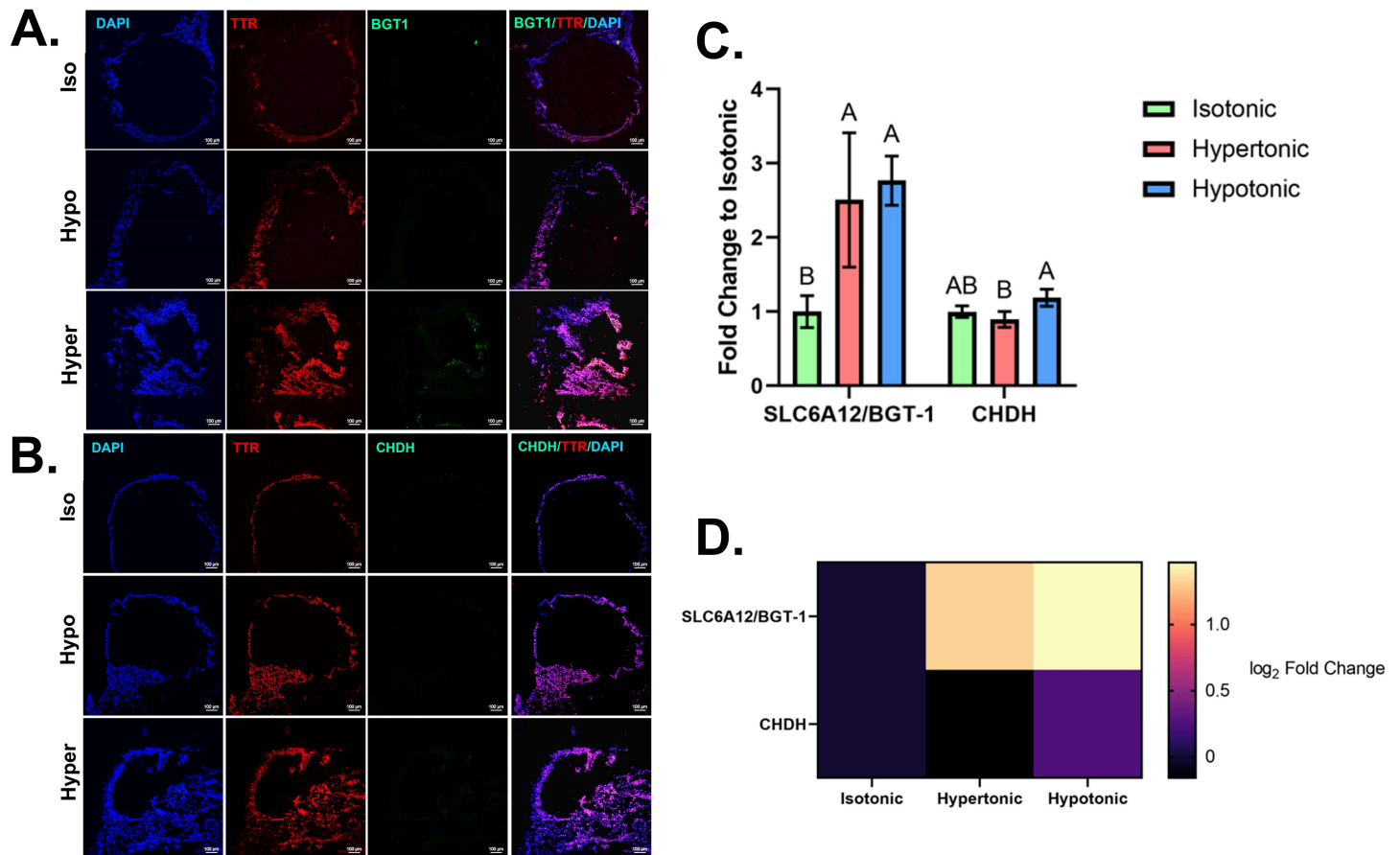


Figure 5

Betaine regulation under osmotic stress (iso, hyper, and hypo). (A – B) BGT1 (A, green) and CHDH (B, green) expression when co-stained with TTR (red) and DAPI (blue) in the CSF-like fluid pockets of ChP brain organoids. Scale bar = 100 μ m. (C – D) Fold change (C) and $\log_2$ fold change heat map (D) of BGT1 and CHDH gene expression after hypertonic and hypotonic treatment. (C: $n = 4$, 1-Way ANOVA with Tukey's *post-hoc*, SLC6A12/BGT-1: $p = ** = 0.0037$, CHDH: $p = ** = 0.0083$, treatments compared to isotonic; all data mean $\pm$ S.D.).

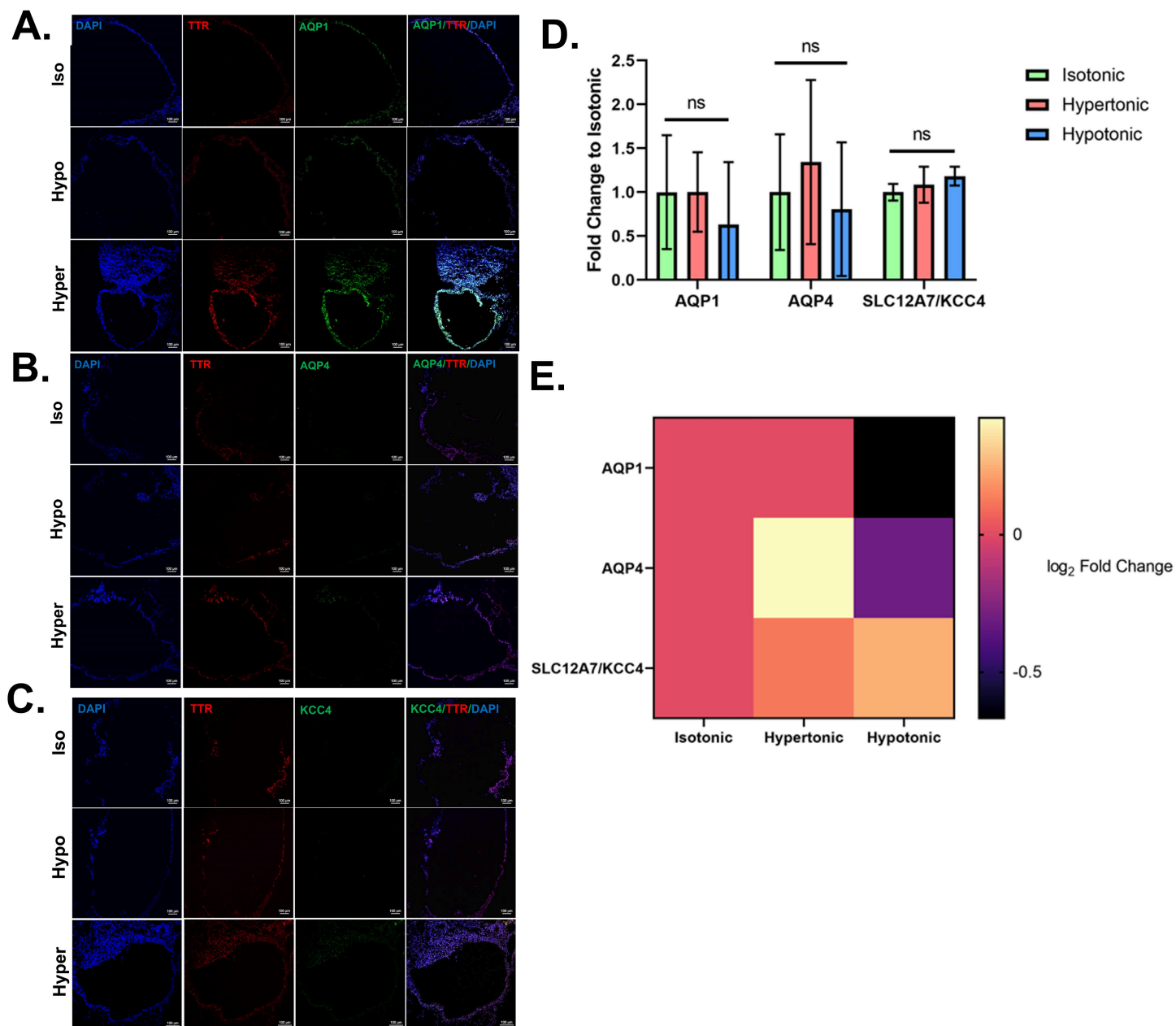


Figure 6

Transport channel regulation under osmotic stress (iso, hyper, and hypo). (A – C) AQP1 (A), AQP4 (B), and KCC4 (C) – all green – expression when co-stained with TTR (red) and DAPI (blue) in the choroidal epithelial layer of ChP brain organoids. Scale bar = 100 μ m. (D – E) Fold change (D) and log₂ fold change heat map (E) of transport channel gene expression after hypertonic and hypotonic treatment. (D: n = 4, 1-Way ANOVA with Tukey's post-hoc, AQP1: p = n.s. = 0.6310, AQP4: p = n.s. = 0.6417, SLC12A7/KCC4: p = n.s. = 0.2531, treatments compared to isotonic; all data mean $\pm$ S.D.).

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