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DOI:
10.1177/0271678X18776226

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citation for published version (APA):

Published in:
Journal of Cerebral Blood Flow and Metabolism

Citing this paper
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Running header: Cerebral folate and congenital hydrocephalus

Alicia Requena Jimenez, Naila Naz and Jaleel A. Miyan

Faculty of Biology, Medicine & Health
Division of Neuroscience & Experimental Psychology
The University of Manchester
3.540 Stopford Building
Oxford Road
Manchester M13 9PT, UK

Author for correspondence: Dr Jaleel Miyan

Faculty of Biology, Medicine & Health
3.540 Stopford Building, Oxford Road
Manchester M13 9PT. UK
Email: j.miyan@manchester.ac.uk
Tel: +44 161 306 4205

Abbreviations: Cerebrospinal fluid (CSF), Hydrocephalus (HC), 5-Methyl-Tetrahydrofolate (5mTHF), Tetrahydrofolate (THF), Folate Receptor Alpha (FRα), Proton Coupled Folate Transporter (PCFT), 10-Formyl-Tetrahydrofolate dehydrogenase (FDH), Hydrocephalic Texas (H-Tx) rat.
ABSTRACT:

Hydrocephalus (HC) is an imbalance in cerebrospinal fluid (CSF) secretion/absorption resulting in fluid accumulation within the brain with consequential pathophysiology. Our research has identified a unique cerebral folate system in which depletion of CSF 10-formyl-tetrahydrofolate-dehydrogenase (FDH) is associated with cortical progenitor cell-cycle arrest in hydrocephalic Texas (H-Tx) rats. We used tissue culture, immunohistochemistry, in-situ PCR and RT-PCR and found that the in-vitro proliferation of arachnoid cells is highly folate-dependent with exacerbated proliferation occurring in hydrocephalic CSF that has low FDH but high folate-receptor-alpha (FRα) and folate. Adding FDH to this CSF prevented aberrant proliferation indicating a regulatory function of FDH on CSF folate concentration. Arachnoid cells have no detectable mRNA for FRα or FDH, but FDH mRNA is found in the choroid plexus (CP) and CSF microvesicles. Co-localization of FDH, FRα and folate suggests important functions of FDH in cerebral folate transport, buffering and function. In conclusion, abnormal CSF levels of FDH, FRα and folate inhibit cortical cell proliferation but allow uncontrolled arachnoid cell division that should increase fluid absorption by increasing the arachnoid although this fails in the hydrocephalic brain. FDH appears to buffer available folate to control arachnoid proliferation and function.

Key words: Hydrocephalus, arachnoid, FDH, FRα, folate

Impact statement: The cerebral folate handling system, involving both FDH and FRα, is vitally important for normal development of the brain and presents a potential target for preventing hydrocephalus and other neurodevelopmental conditions.
INTRODUCTION

Congenital hydrocephalus (HC) is the outcome of developmental CSF flow obstruction and/or drainage insufficiency. It is a worldwide neurological condition with a reported global incidence of 1:500 live human births.\(^1\) It is associated with ventriculomegaly and, postnatally, raised intracranial pressure\(^2,3\) leading to neurological deficits, brain damage and death. In spite of more than 100 years of research, the specific cause of CSF obstruction is still a subject of study.\(^4\)

The hydrocephalic Texas (H-Tx) rat model, with many comparable brain abnormalities to human congenital HC\(^5,6\), has been invaluable in investigating early events of this disorder.\(^7\) In fact, between embryonic days 17 and 18, affected H-Tx fetuses share comparable cerebral cortex abnormalities with human fetal HC at Carnegie’s stage 23 (56 days).\(^8,10\) Two main areas of the brain are critical in early-onset HC: the first is the choroid plexus (CP) which is the site of cerebral CSF production and also presents the blood-CSF barrier (BCB), and the second is the leptomeninge arachnoid (or arachnoid membrane) across which CSF exits the brain at specific sites and enters venous and lymphatic drainage pathways, and which thus represents the CSF-blood barrier (CBB). These two structures, forming production and drainage sites for CSF, start developing around embryonic day 7.5 and 13 in rats respectively.\(^11,12\) The functionality of both barriers is crucial for adequate CSF dynamics, prevention of hydrocephalus and development of the CNS.\(^13,14\) The present study aims to provide more insight into the complex etiopathology of congenital HC that might benefit from a preventive treatment with suitable maternal supplementation to avoid the requirement for invasive treatments (shunting) associated with a high risk of failure and death in the first two years of life.\(^15\)

An H-Tx rat colony derived from a spontaneous mutation in a normal, presumed Sprague Dawley (SD) rat colony and then maintained through brother-sister mating to produce at least 26% of the progeny developing HC\(^16\) was used in this study. HC in these rats is associated with a stenosis of the aqueduct of Sylvius and morphological changes of the subcomissural organ\(^7\) but is thought to be primarily caused by a drainage insufficiency at the arachnoid. Although the precise mechanism leading to CSF obstruction is still awaiting characterization, we believe that a primary fault in cerebral folate supply and handling may be responsible\(^43\).

Folate is a crucial nutrient for multiple metabolic pathways. Its deficiency or imbalance is reported to have severe consequences in the developing embryo-fetus, not least through its relevance in cell division.\(^17,20\) 5 methyl tetrahydrofolate (5mTHF) is the prevailing active form of folate in dietary sources and the main type found in the bloodstream; hence it is also the folate that is predominantly transported into peripheral tissue for cellular metabolism.\(^21\)

Within the brain, among different forms of folate, only 5mTHF can cross the blood-brain barrier (BBB) freely.\(^22\) It is well established that in contrast to leaky vessels in peripheral organs\(^23\) the BBB restricts entry of polar molecules into the brain\(^24,26\) but these can be transported into the brain via specific transporters expressed in brain endothelium and choroid plexus epithelium under physiological or pathological conditions.\(^27,28\) Additionally, it
is known that several BBB peptide transport mechanisms exist, for example, receptor-mediated, adsorptive-mediated, carrier-mediated and non-specific passive diffusion.\(^{29}\)

Maternal plasma 5mTHF binds to FRα, a major folate transporter,\(^{14}\) and is translocated across the placental membranes to the fetal side\(^{30,31}\) reaching the fetal choroid plexuses (CP) for transport across the blood-CSF barrier into the CSF,\(^{14,32}\) where it promotes development of the developing brain.\(^{33}\) FRα is highly expressed in placental trophoblast at early gestational days\(^{31,34}\) but not in the CP.\(^{35}\) Placenta and fetal brain studies in mice revealed a peak in FRα expression in first trimester placenta compared to CNS tissue, where FRα expression is lower at any time point in development. These facts show the importance of FRα expression during pregnancy in comparison with other folate transporters.\(^{14}\) Disruption of FRα alleles is embryonically lethal at about the time of the neural tube closure.\(^{36}\) In this regard, the function of FRα in providing 5mTHF across placenta and CP is restricted to embryonic and fetal development\(^{37}\) and 5mTHF-FRα mediated transport prevails over other transport mechanisms in situations of high 5mTHF demand.\(^{13}\)

In spite of FRα being described as the principal transporter for 5mTHF in the brain, we\(^{38}\) and others\(^{39-41}\) have previously reported that FDH also has a role in folate handling as well as metabolism.\(^{42}\) Past in vitro work\(^{39}\) established that FDH regulates the folate-mediated one-carbon metabolic cycle, which is defective in congenital HC and linked to depletion of FDH in CSF.\(^{43}\) These findings all suggest a key role of FDH in controlling folate metabolism and folate levels in CSF as well as brain tissue storage\(^{44}\) permanently removing one-carbon units from the intracellular and extracellular pool.\(^{39,40}\) Consequently, abnormal FDH expression at tissue level and in CSF are associated with changes in brain cell proliferation\(^{43,45}\) a reduction in cortical thickness associated with cortical progenitor cell cycle arrest, and reduced cerebral cortex development.\(^{10}\) Hydrocephalic cell cycle blockade was also reflected in changes in CSF composition related to the folate metabolic cycle suggesting that modifications in folate bioavailability to the developing brain may be a primary fault leading to congenital HC.\(^{43}\)

Recently, it has been reported that CP sheds exosomes from the apical membrane that shuttle FRα-5mTHF complexes into the CSF.\(^{13}\) Because FDH is present in the CP and CSF and has affinity for 5mTHF, it is reasonable to propose that FDH may also have a role in 5mTHF transport, along with FRα within the CP as well as through the CSF to supply the cerebral cortex by means of the exosomes described previously.\(^{13}\) Such a mechanism for FDH-5mTHF transport has been partially characterized and identified in radial glia,\(^{38}\) but not in CP, and the full mechanism needs further investigation, which is the object of this research.

This study aims to assess FDH and FRα expression in the brain, their affinity with 5mTHF and potential role in folate transport within CP. Moreover, we aim to elucidate the folate-dependent arachnoid dysfunction as a consequence of excess 5mTHF provision by elevated soluble FRα released into CSF and the effects of low CSF FDH in hydrocephalus.
MATERIAL AND METHODS

Animal colony

Animal experiments were performed at the Biological Services Unit at the University of Manchester. All experiments were sanctioned by The Home Office Animals (Scientific Procedures) Act (1986) and Inspectorate under scientific and ethical review of a Project Licence (70/8025). All experiments were also regulated by The University of Manchester Ethical Review Committee and conformed to ARRIVE guidelines. Sprague-Dawley (SD) rats were used as controls as they are thought to be the origin of the H-Tx strain and have the same gestation time. Whereas the SD colony was maintained through random pair mating, the H-Tx colony was subjected to brother-sister matings to establish a breeding colony that normally gave rise to 23% of newborn pups having HC. Generation of timed matings was achieved by housing males and females in the same cage where attached trays allowed vaginal plugs to be found when successful mating took place. We considered the day on which the vaginal plug was found as day zero. Timed matings were arranged to produce fetuses of the specific age required for our experiments [embryonic day (ED) 18]. All rats were kept in a 12h:12h light:dark cycle and had free access to food and water. Their diet was based on a standard BK universal rat diet which contains folic acid at 3.2 mg/kg. Pregnant rats were euthanized by carbon dioxide inhalation, and fetuses were removed from the uterus and placed directly onto ice. Fetuses were fixed in cold 4% paraformaldehyde in phosphate buffer (pH 7.4) overnight. The fixed fetuses were cryoprotected with 10% sucrose for 24h, followed by 20% of sucrose for 48h in phosphate buffer, pH 7.4. After cryoprotection, the fetuses were frozen in isopentane cooled with dry ice. 15 µm thick frozen sections were cut on a Leica CM1900 cryostat (Leica, Milton Keynes, UK) and collected onto gelatine-coated glass slides that were left to dry overnight before storage at -20°C for later processing and staining.

H-Tx fetuses were distinguished as affected or unaffected based on morphological analysis of brain cryosections and analysis of cortical thickness as well as lateral ventricle gross size. All fetuses were processed, and the obtained incidence data were statistically analysed by Chi square test.

CSF collection

Fetal CSF was collected by insertion of a fine glass pipette into the cisterna magna and lateral ventricle of SD and affected fetuses. Aspiration was avoided because of the risk of contamination with blood. All CSF samples were centrifuged at 6,500 rpm for 10 minutes to remove contamination with cellular debri and the supernatant stored at -80°C.

Arachnoid tissue processing and identification

Fetal arachnoid membrane was isolated under the dissecting microscope and used within 24 hours post-mortem. Briefly, arachnoid tissue was incubated with primary antibodies and markers of arachnoids i.e.: Zone occludens-1 (ZO-1 Invitrogen, Paisley, UK,) and cytoplasmic-specific protein Cytokeratin 18 (Abcam, Cambridge, UK) for 1 hour, washed
with washing solution, and incubated with secondary antibodies Alexa Fluor 488 conjugated goat anti-rabbit (green staining) or Alexa Fluor 594 donkey anti-mouse (red staining) (Invitrogon, Paisley, UK) at 1:500 dilution for 1 hour. Finally, cells were counterstained with DAPI, and mounted with antifade reagent (Prolong Gold, Invitrogon, Paisley, UK). Images were captured with a Leica DMLB fluorescence microscope connected to a Coolsnap digital camera and Metaview software v5. Image analysis was using ImageJ (NIH) v1.36b.

Maintaining primary arachnoid cell culture: The colourless and web-like semi-transparent arachnoid membranes were removed from brains at ED 18 under the operating microscope. The membranes were cut into 2 mm sections and plated in 25-cm² Falcon flasks with complete medium (Dulbecco’ modified Eagles’ medium (DMEM). DMEM medium was supplemented with 20% (v/v) fetal bovine serum, 100U/ml penicillin and 0.1 mU/ml insulin. The flasks were maintained at 37°C in a 100% humidity incubator with 5% CO₂. After 24h incubation, the medium was replaced with fresh medium and this was again changed every 4 days. Arachnoid cells were passaged on day 15-20 from the bottom of the flask. Once the media was discarded, the arachnoid cells were washed three times with Hanks’ buffer and digested in 1:1 (v/v) dilution containing 0.30% (w/v) trypsin at room temperature for 10 minutes. Digestion was stopped with complete medium to obtain a cell suspension concentration of 4x10⁵ cells/ml. Arachnoidal cell proliferation achieved total confluence after 72 hours.

Experimental groups: The culture was then divided into subgroups: negative control (cells grown in media exclusively), control (cells grown in normal CSF obtained from normal animals), group 1 (cells grown in hydrocephalic CSF i.e. low FDH but high 5mTHF and high FRα) group 2 (high 5mTHF) and group 3 (CSF with high 5mTHF but normal levels of FDH). Control and group 1 recapitulated the natural environment where arachnoid cells proliferate and differentiate in normal and abnormal conditions respectively. Since arachnoid cells and radial glia share embryonic origin and stem cell properties and radial glia is also highly 5mTHF-dependent for cell division, primary radial glia cell culture was used as positive control and media without folates as negative control.

Treatment of primary cell culture: After 15-20 days of growth at 37°C in 5% carbon dioxide, arachnoid cells were treated with 50% normal or hydrocephalic CSF diluted in complete media, as well as media either containing 5mTHF at 10-4 M alone or in combination with either FDH or FRα at 10-5 M. 5mTHF, FDH and FRα concentration values were chosen according to estimations for CSF previously published. Active FDH was kindly provided by Dr. S Krupenco, Medical University of South Carolina, USA, and commercial FRα (R&D systems) and 5mTHF (Sigma-Aldrich) were also used.

DAPI staining of primary arachnoid cell cultures after treatments: DAPI was used for nuclear double stranded DNA staining (Invitrogon, Paisley UK) on cultured arachnoid cells exposed to each different treatment. Cells at 4 x 10⁵ cells/ml were added to 24-well plates containing 10 mm glass round coverslips (VWR International, Ltd.). Cells were stained and coverslips mounted with antifade reagent (Prolong Gold, Invitrogon, Paisley, UK). Images
were captured with a Leica DMLB fluorescence microscope connected to a Coolsnap digital camera.

**Analysis of cell proliferation:** Cell proliferation was measured using the fluorescence PrestoBlue cell viability reagent kit (Invitrogen, Paisley, UK). Fluorescence signal was measured on a Magellan 200 fluorimeter (TECAN, UK). Data was statistically analyzed using a two-ways ANOVA with Bonferroni post hoc test.

**Immunofluorescence staining**

Immunohistochemistry was performed as described previously. Cryosections were probed with rabbit anti-rat FDH (a gift from Dr. S. Krupenco), mouse anti-rat 5mTHF (Millipore), sheep anti-mouse FRα (R&D Systems), and CD 133 Abcam Cambridge, UK) diluted at 1:1000, 1:2500, 1:20 and 1:100 respectively. Images were captured on a Leica DMLB fluorescence microscope connected to a Coolsnap digital camera (Princeton Scientific Instruments, Monmouth Junction, New Jersey, USA). Images were analysed for level of fluorescence using Image J 1.36b image processing software (National Institute of Health; http:rsb.info.nih.gov/ij).

**Immunofluorescence quantification**

Tissue sections were captured on a Leica DMLB fluorescence microscope connected to a Coolsnap digital camera (Princeton Scientific Instruments, Monmouth Junction, New Jersey, USA). Constant exposure times (500 milliseconds) were kept within and between stains. Images were analysed for level of fluorescence using Image J 1.36b image processing software (National Institute of Health; http:rsb.info.nih.gov/ij). At least 10 images per experimental condition were imaged. The colour channel corresponding to the fluorophore used (green or red) from the source multichannel/composite image was selected, and the area of interest (fluorescent area) to be included in analysis was marked out using the “ROI” plugin. The “Threshold” plugin was used to determine the minimum and maximum signal/number of pixels in the region of interest and a value with intensity above the minimum threshold reading was chosen arbitrarily. The threshold value was maintained within and between stains. All regions of interest were counted and averaged using the tool ROI manager. Pixels were converted to area in microns squared (100x magnification, 1 micron=1 pixel). To avoid artificial inflation of positive staining in tissue, areas of fluorescence above the threshold value, but less than 10µm² in area, were considered too small to be cells and were not included in the analysis. Total area above the threshold was calculated against a total area of analysis (10⁵ µm²). Area/pixels above the threshold (area positive) were recorded and expressed as density of positive staining within the total area analysed. Density was calculated using the formula shown below:

\[
\text{Fluorescence density} = \frac{\text{Area positive (fluorescence above threshold)} \times 10^5 \mu m^2}{\text{Total area analysed}}
\]
FRα and FDH mRNA quantification in arachnoid tissue extracts using real time quantitative PCR (RTqPCR)

Real-time quantitative PCR (RTqPCR) was used to measure ALDH1L1 mRNA expression in normal and hydrocephalic arachnoid. Omission of reverse transcriptase was used as negative control. cDNA was amplified using SYBR Green Jump Start master mix following the company’s instructions (SIGMA). cDNA integrity was confirmed by β-actin amplification. Amplification of tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein (YWHAZ) mRNA (Table 1), a stable reference gene expressed in brain, confirmed cDNA input was comparable between normal and hydrocephalic cerebral cortex samples. Specific primer sequences for cDNA amplification (Table 1) were designed from GenBank sequences using Roche ProbeFinder for rat version 2.49 (www.rochедиagnosticss.qpcr.probefinder.com). mRNA expression was quantified using Step-One AB Aplied Biosystems with normalization reference to gene. All reactions were run in triplicates using ROX as passive reference dye. PCR conditions were as follows: initial denaturation at 95°C x 15 min, followed by 40 PCR cycles of denaturation at 94°C X 15 sec, annealing for 30 sec at optimal annealing temperature (T_a in Table 1) and extension at 72°C X 30 sec. PCR reaction efficiency approximated to 100% for all genes. Relative quantification of cDNA amplification (mRNA expression) was carried out using the comparative method following Step-One AB Applied Biosystems software version 2.2 instructions.

In-situ Reverse Transcription PCR

The in-situ RTPCR protocol was performed as described by GJ Nuovo in 1997. Negative control (no primers), positive control (liver and placenta tissue) and test sections were all placed on the corresponding slides; microslides, superfrost ultraplus (VWR International Ltd.) A Leica DMLB fluorescence microscope coupled to a coolsnap digital camera (Princeton Scientific Instruments, New Jersey; USA) was used to take images of transmitted light. All images were transferred to Image J software (version 1.37a; NIH, MA, USA). FDH cDNA with accession number: NM_022547.1 was amplified with sense primer TCGACACTCAAC and antisense primer ACTTCAGGA.

Confocal microscopy

A Leica SP5 acousto-optical beam splitter confocal microscope (Leica, Milton Keynes, UK) was used for imaging of 4’6-diamidino-2-phenylindole (DAPI) and Rhodamine. 405/450 excitation/emission laser lines for DAPI and 510/590 excitation/emission laser lines for detection of FDH mRNA were used respectively. Images of transmitted light were transferred to Image J software (version 1.37a; NIH, USA).

Data Analysis and statistics

The data was analysed using GraphPad Prism v6. Two-ways analysis of variance ANOVA was used when more than one factor/treatment was studied on different cell populations, and one way analysis of variance when only one factor was studied to determine if there were
significant differences between the groups. Post hoc tests were performed using Bonferroni to compare all experimental groups.

RESULTS

Arachnoid cell growth is 5mTHF-dependent

Figure 1 shows successful culture, growth (Figure 1A-C) and characterisation (Figure 1D-F) of primary arachnoid membrane cells taken from rats. Proliferation in different culture conditions was measured using the nuclear stain DAPI (Figure 1G-K). Arachnoid cell growth was at its least in negative control (media only) groups as expected (Figure 2). Control groups (normal CSF with normal FDH, FRα and 5mTH) showed an increased growth of arachnoid cells in comparison to negative control groups indicating that FDH, FRα and 5mTH are important for arachnoid cell growth. Group 3 (FDH at normal levels but high 5mTH) showed increased cell growth compared to control group indicating 5mTHF can increase the proliferation of arachnoid cells. There was further increased in cell proliferation in group 1 (abnormal CSF with low FDH but high 5mTHF) indicating that in reduced or no FDH there is more 5mTHF availability to cells for proliferation. The maximum cell growth was observed in group 2 (cell grown in high levels of 5mTHF) indicating the importance of 5mTHF for arachnoid cell proliferation. Analysis of variance (two-ways ANOVA) with Bonferroni post hoc test revealed significant differences between all columns, ***p<0.001. These data suggest that arachnoid development is stimulated by 5mTHF. Moreover, under hydrocephalic conditions of increased FRα/5mTHF and decreased FDH levels, arachnoid cell division is abnormally high. Additionally, arachnoid cell growth with FDH at normal CSF levels in the presence of abnormal 5mTHF concentration caused a halt in cell division, which suggests that FDH regulates 5mTHF levels in normal CSF. On this basis, low FDH in hydrocephalic CSF results in accumulation of 5mTHF in CSF.

There is no FRα and FDH expression in the embryonic arachnoid

Placenta and liver were used as positive control tissue for relative quantification of FRα and FDH mRNA respectively, using traditional Real Time PCR (RTPCR), and all samples included were normalized to the calibrator (Figure 3). FRα mRNA expression in placenta was remarkable and comparison of FRα expression levels between placenta and arachnoid tissue demonstrated FRα synthesis in the arachnoid is negligible. Similarly, expression of mRNA for FDH in liver was significantly high but undetected in the arachnoid. mRNA analysis for all genes was performed simultaneously, and all samples amplified for β-actin. The data indicated a lack of FRα and FDH expression in arachnoid.

FDH is constitutively expressed by the CP of normal and hydrocephalic brain
In situ reverse transcription PCR (In situ RTPCR) allowed identification of FDH mRNA in radial glia cells (intense white staining in the form of clusters in Figure 4, image A, and selected magnified area; image B). FDH’s presence in radial glia was expected; however, surprisingly, FDH mRNA was also noticeable in the CP of lateral ventricles in normal brain (intense white staining in image D), and in hydrocephalic brain (discernible white staining in image E). These results emphasize the suitability of in situ RTPCR to detect mRNA, corroborate the location and expression of FDH, and demonstrate for the first time FDH gene expression in CP. Interestingly, FDH mRNA was also seen in vesicle-like structures (image E white arrows indicating positive vesicles). The data indicate presence of FDH mRNA in CP and vesicles around CP.

In situ RTPCR positive tissue control (liver) was used for determination of PCR integrity and suitability of the primers used for amplification. Inhibition of genomic DNA amplification was avoided by suitable primer design as described in material and methods. DNase treatment was also carried out to avoid any potential genomic DNA amplification. Negative controls for brain and liver tissue FDH mRNA amplification (lack of forward or reverse primer), and negative controls for DNase effect (no DNase treatment for inactivation of genomic DNA) were included in parallel to gene amplification, resulting in a null signal (data not shown).

FDH protein and FRα protein co-localize in normal and hydrocephalic CP

Fluorescence immunostaining of brain sections (Figure 5) indicated FDH (intense green staining) and FRα/5mTHF (intense red staining) in normal CP (A, B and C and A’, B’, and C’ images), and abnormal CP (D, E and F and D’, E’ and F’). FDH co-localization with FRα and 5mTHF (intense yellow staining) suggests a role for FDH in 5mTHF transport with FRα. The specificity of all antibodies was confirmed by lack of detectable stain in negative controls (H and I for FDH and 5mTHF, and H’ and I’ for FRα and 5mTHF). This data indicate that FDH is colocalized with 5mTHF and FRα within CP indicating a potential involvement of FDH in transport of 5mTHF alongside FRα.

FRα protein is overexpressed in the hydrocephalic CP and correlates with 5mTHF but not with FDH

Fluorescence FRα staining quantification analysis of hydrocephalic tissue (Figure 6) showed a statistically significant increase in fluorescence mean density value (58438±893) when compared to mean control values (20605±10290), (p≤0.01). Similarly, 5mTHF staining of abnormal brain exhibited a remarkable increase in signal (42717±3389) in comparison with controls (8326 ±6244), (p≤0.01). In contrast, a minor decrease in FDH fluorescence optical values in abnormal brain (55326±566) was detected as opposed to mean control values (50266±9807). However, this data was not statistically significantly different after analysis of
variance (p≥0.05). This data indicate that FRα protein is increased in the embryonic hydrocephalic CP and correlates with 5mTHF but not with FDH.

DISCUSSION

Abnormalities in CSF composition separately and/or in combination with impaired secretion, circulation and absorption have all been linked to different neurological conditions. This study focuses on sites of CSF production (CP) and drainage (arachnoid) to understand more about the multifunctional protein FDH which we previously reported to be absent or reduced in hydrocephalic CSF. In this study we investigate FDH for its recently reported affinity for 5mTHF in the cerebral cortex and its relationship with FRα, the major folate transporter.

In the current study we have reported an in-vitro FDH folate-regulatory function controlling 5mTHF fluctuations. We demonstrated that arachnoid tissue growth is overstimulated in in-vitro culture mimicking hydrocephalic conditions when cells are bathed by CSF containing high 5mTHF/FRα and low FDH levels. Our findings agree with, reporting how FDH downregulation has an impact on high proliferation of tumour cells and cancer. However, this is in stark contrast to the inhibition of cortical cell proliferation by hydrocephalic CSF. It has been previously reported that maturation of certain brain cell-types requires a decrease in the percentage of mitotic cells but augmentation of the cell cycle length for acquisition of normal organ function. The present study raises the possibility that maturation of the leptomeninge arachnoid and acquisition of normal function is finally adjusted to the specific levels of FDH in CSF and that FDH fluctuation may have a negative impact on cell division, development and maturation of arachnoid cells.

In relation to these findings, we suggested that the low levels of FDH in hydrocephalic CSF are detrimental for regulation of 5mTHF levels, resulting in elevated 5mTHF. Because CSF bathes the arachnoid providing 5mTHF for its further development, abundant available 5mTHF in CSF may lead to overstimulation of arachnoid cell division that results in changes in tridimensional structure, and somehow, in a dysfunctional arachnoid tissue that is unable to perform its remarkable function of transporting CSF across the CSF-blood barrier and thus maintaining CSF flow and pressure homeostasis. These events may contribute to CSF obstruction and consequently may lead to congenital HC as has been report previously. It is interesting to speculate that CSF folate imbalance may also be responsible for arachnoid granulations observed in ageing human brains considering the correlation of decreased CSF volume production, perhaps indicating drainage insufficiency, associated with dementia for example.

It is not unexpected to find FDH and FRα in embryonic arachnoid cells if their division is highly dependent on 5mTHF. However, we could not detect any mRNA expression for FRα or FDH in the arachnoid at ED 18 suggesting these transporters bring folate to the arachnoid via the CSF pathway and are not manufactured within the arachnoid as also reported previously. FRα is known to exist in soluble form in body fluids and we detected it in our
CSF samples (see Supplementary Figure A), hence, we propose that soluble FRα-5mTHF complexes reach the cell membrane of the arachnoid where they may bind to transmembrane receptors other than FRα through glycoporphosphatidylinositol, the known mechanism on the apical membrane of the CP.58,59 This may be similar to interleukin that is reported to associate with cell-surface receptors other than their corresponding transmembrane receptors to confer novel ligand sensitivity to cells.60 The ligand activates, in this fashion, related signalling pathways without the need of constitutively expressing Interleukin transmembrane receptor.60-62 Another plausible explanation for the absence of FRα expression by the arachnoid is that in situations of high 5mTHF demand and/or high 5mTHF concentration in CSF, as in HC, other ubiquitously produced folate transporters such as organic transporters (Reduced Folate Carrier, RFC) may mediate 5mTHF uptake.13,14 This is in accordance with our findings that arachnoidal tissue is highly dependent on 5mTHF for cell division. Lack of FRα synthesis in arachnoid tissue could also reflect the importance of the placenta in the biosynthesis of FRα to deliver folate directly to the embryonic brain.37 The present study supports the idea that satisfactory placental function is paramount for 5MmHFKFRα transport at early gestational days as reported previously.31,34 In this respect, our results also suggest that placental function may be impaired in congenital HC, resulting in soluble FRα overexpression, probably in response to the decreased levels of FDH expression in the brain.38,43 This idea is also sustained by ultrasonographic placenta examination studies demonstrating that the hydrocephalic placenta is structurally abnormal.53

We previously demonstrated that FDH binds with FRα and translocates 5mTHF into the radial glia of the cortex.38 In this study we report that FDH is associated with FRα for folate transport not only in cortex but also in/across CP. The current study identified FDH mRNA in CP at embryonic day (ED) 18 when high volume CSF production has begun.3 Moreover, a colocalization of FDH protein with FRα protein in CP and in vesicle-like structures close to the CP indicates that FDH interrelates with FRα to transport 5mTHF across CP. These findings imply and extend our understanding of folate transport across the brain. Extracellular vesicles from both CP and radial glia may be working in synergy to transport 5mTHF to the cerebral cortex, and merging of these vesicles cannot be discarded.38

FRα-5mTHF complexes have recently been detected in CP immortalized cell lines (Z310) as well as in CP-associated exosomes in mature brain.51 Moreover, CD133 positive vesicles shedding from radial glia and carrying FDH have been identified extracellularly in the past indicating a radial glial/stem cell origin for these. We propose that FDH may be part of the transduction signal mechanism suggested by13 which is required for FRα-5mTHF-exosome release into the radial glia.13 Moreover, it seems likely that the radial glia is responsible for the decrease in FDH release into CSF as FDH expression in normal and abnormal CP was similar while CSF levels are greatly reduced in HC38 with CD133 positive vesicles containing FDH shedding from radial glia66 and carrying FDH into the CSF.38,65 CP has a very distinctive structure and location in the brain (the ventricles) making it not very crucial to use a CP specific marker in the present study. However, the nature of FDH
positive vesicles needs further clarification to shed light on their different origins and relationships.

Fluorescence analysis demonstrated increased levels of FRα and 5mTHF in H-Tx CP tissue. 5mTHF levels were also increased in hydrocephalic CSF, but although detected, we could not confirm FRα concentrations in hydrocephalic CSF and thus whether it follows. An inverse relationship to folate as previously described, however, we found that FRα presence is increased in the CP in situations of high extracellular 5mTHF levels (hydrocephalic CSF). Thus, we propose that FRα abundance in the CP is possibly a mechanism to increase 5mTHF provision in response to low FDH expression in CSF since FDH and FRα seem to be both necessary to transport 5mTHF with FDH having additional regulatory functions to store folate intracellularly. We suggest that FDH is also regulating levels of 5mTHF extracellularly, in CSF, by binding to folate and sequester it in situations of high 5mTHF concentration. In this manner, increased levels of 5mTHF may be explained by the low levels of FDH in H-Tx CSF hindering the appropriate and controlled storage of 5mTHF.

**Clinical significance**: The present study provides more insight into the complex etiopathology of congenital HC that might benefit from a preventive treatment with suitable maternal supplementation to avoid the requirement for invasive treatments (shunting) associated with a high risk of failure and death in the first two years of life.

**Acknowledgements**

We thank the BSU for technical assistance and to the Bioimaging core facility for access to confocal microscopy. This research was funded by The Charles Wolfson Charitable Trust (JAM) as well as an MRC Doctoral Scholarship to ARJ.

**Authors' contributions**

JAM and ARJ conceived and designed the study. ARJ carried out all experimental work and data analysis. NN critically commented on the study and with ARJ and JAM worked on the manuscript writing and editing.

**Conflict of interest**

The authors declare that they have no competing interests.

**Supplementary material**

Supplementary material for this paper can be found at [http://jcbfm.sagepub.com/content/by-supplemental-data](http://jcbfm.sagepub.com/content/by-supplemental-data)
Reference List


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Figure Legends

Figure 1.

Bright-field microscopy of Sprague Dawley cultured arachnoid (A, B and C) at gestational day 18. Extracted arachnoid fragments (dashed black arrow) after 24 hours growth; (A), tissue-like scaffolds at 48 hours (B) and polygonal cell monolayer at 72 hours (C). White arrows indicate large nuclei. Immunohistochemical staining shows arachnoid markers in (D, E, and F). Cells are linked together by zona occludens-1 (green fluorescence); (white arrows); (D). Cytokeratin 18 (red fluorescence); (black arrows); (E). Merged channels (F). DAPI staining after treatment with normal CSF effect (G), x20. Hydrocephalic CSF (H), x20; 5MTHF (I), X20, and 5MTHF + FDH (J), x20. DAPI staining of Sprague Dawley cerebral cortex stem cells/control; (K), x40. Scale bar: 50 µm.

Figure 2.

Figure 2. CSF, 5MTHF and FDH effect on primary arachnoid cell culture and cerebral cortex progenitor/stem cells. Growth in media without 5MTHF (black column). Cell proliferation with normal CSF (dark pink pattern) versus hydrocephalic CSF (pale pink pattern). Single 5MTHF impact on cell division (purple column) versus 5MTHF plus FDH (dotted column). Stem cell proliferation with normal CSF (black square pattern). Data are presented as (%) change in cell change ± SEM obtained after normalization of optical density values using a fluorescence cell viability kit as described in Material and Methods. Analysis of variance (ANOVA) revealed high significant differences (**p <0.001)

Figure 3.

Quantification of mRNA expression (ΔΔCt method) for FRα versus FDH in normal arachnoid tissue extracts at embryonic day 18. Circles represent placenta tissue control for FRα. Triangles (upwards) represent FRα mRNA expression. Squares indicate liver tissue control for FDH. Triangles (downwards) mRNA FDH expression. N=6. mRNA expression was normalized to tyrosine 3-monooxigenase/tryptophan 5-monooxygenase activation protein (YWHAZ). Data are shown as scatter plot with the media represented by the horizontal line. P*<0.05

Figure 4.

Figure 4. Fluorescence “in situ” RTPCR. FDH cDNA tissue distribution in coronal brain sections (15 µm) in normal (SD) and hydrocephalic brain (Tx) at embryonic day 18. Positive labelling was detected in animal control (SD) radial glia (RG) as shown in (A), (X20) and magnified area, (B), (x40). RG stained with CD133 marker, (C), (x100). Liver tissue + control, (D), x40. FDH in CP of SD, (E), x20. FDH in CP of Tx, (F), (X20). Confocal image of FDH cDNA rhodamine labelled (red) + DAPI nuclei staining (blue), (G). Blue nuclei indicates negative FDH expression from genomic DNA and red positive staining around nuclei represents detection of cDNA exclusively. (-) control, (H). cDNA Fluorescence quantification, (I). Analysis of variance (ANOVA) revealed non-significant differences between SD and Tx. Scale bar 100µm.
Figure 5.

FDH, FRα and 5MTHF fluorescence immunolocalization within the choroid plexuses of lateral brain ventricles. FDH (green), FRα (red right images), 5MTHF (red; left images), and merged images (yellow). A, B and C (right), and A’, B’ and C’ (left) images show staining in normal CP whereas D, E and F (right) and D’, E’ and F’ (left) images indicate positive signal in Tx CP. Scale bar 40µm. Confocal sagittal images of brain ventricles show cerebral cortex (CC) and CP staining in G (FDH), H (FRα) and I (merged channels). Schematic representation of left (LLV) and right (RLV) sagittal brain ventricles sections. K and L are negative controls. Scale bar 100µm

Figure 6.

Choroid plexus (CP) immunohistochemical fluorescence quantification in Sprague Dawley control (SD) and hydrocephalic Texas rats. Fluorescence signal measurements for FDH (green), FRα (red squares) and M5THF (red lines) was analysed as described in material and methods. The density of immunohistochemical staining was calculated per unit of area of section measured for CP in each category. Data from a minimum of 6 sections from each brain were averaged and presented as mean ±SEM for all embryos in each group (6), calculated from all litters (6). Analysis of variance (ANOVA) revealed significant differences between controls and Texas rats for FRα and M5HTF at p≤0.01 but not for FDH, which was non- significant (NS) p>0.05.
Table 1. Gene sequences and annealing temperatures \((T_a)\) for primers targeting FDH and FRα, housekeeping and reference genes.

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<th>Genes (rat)</th>
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254x190mm (96 x 96 DPI)
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