# ROLE OF FOLATES IN NORMAL AND

# HYDROCEPHALIC

# FETAL BRAIN DEVELOPMENT

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**TABLE OF CONTENTS** **Page**

**CHAPTER 2. FDH AND FOLATES ROLE IN BRAIN DEVELOPMENT AT**

**EARLY PREGNANCY: IMPLICATIONS FOR CONGENITAL**

**HYDROCEPHALUS**

**CHAPTER 3. FOLATES ARE CRUCIAL FOR FETAL MENINGEAL ARACHNOID CELL GROWTH: IMPLICATIONS FOR CONGENITAL HYDROCEPHALUS**

**CHAPTER 4. NORMAL VERSUS HYDROCEPHALIC CSF**

**INGENUITY PATHWAY ANALYSIS (IPA) OF PROTEIN PROFILES**

**OBTAINED FROM LCMS**

# CHAPTER 5. CONCLUDING REMARKS, DISCUSSION AND FUTURE AIMS

**Word count:  63,288**

# TABLE OF FIGURES

**CHAPTER 1**

**CHAPTER 5**

# LIST OF TABLES

**CHAPTER 2**

**CHAPTER 4**

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# GENERAL ABSTRACT

Brain Cerebrospinal fluid (CSF) bulk flow is maintained thanks to a balance between CSF secretion from the choroid plexus and CSF absorption by arachnoid villi, where it drains into nearby blood vessels, thereby reaching the general blood circulation. Congenital hydrocephalus starts during the first trimester of pregnancy with impeded CSF flow, and consequent CSF build-up within the brain ventricles. This event is followed by CSF compositional changes, increased intracranial pressure, and, if untreated, brain damage and fetal death. Previous research has revealed a unique folate delivery system which serves the developing cerebral cortex. Abnormal folate provision due to impairment of this system was directly connected to a decrease in a CSF folate enzyme: 10-Formyl-Tetrahydrofolate dehydrogenase (FDH). In light of these findings, low FDH was linked with folate deficiency and the poor cortical development found in congenital hydrocephalus. In this context, investigations were carried out to ascertain whether folates in the presence and absence of the folate enzyme FDH are beneficial for fetal brain development. The current study also aims to investigate the FDH -folate delivery system in the fetal brain in order to understand its role in CNS development and its relationship to currently known folate transport mechanisms (FRα). Furthermore, we hypothesize that folates may prevent congenital hydrocephalus through a re-establishment of CSF drainage and flow circulation at the level of the arachnoid membrane/villi. This assumption implies that the leptomeninge arachnoid may also be dysfunctional in the hydrocephalic brain due to a variation in hydrocephalic CSF composition (folates). Finally, an overall metabolic pathway analysis of the constituents uniquely present in abnormal CSF, hence missing in normal CSF, and vice versa, was carried out to establish associations with suggested activated and inactivated biological processes during congenital hydrocephalus.

# GENERAL INTRODUCTION

## 1.1 OVERVIEW

Congenital hydrocephalus is a worldwide neurological condition with an estimated incidence range of 1/5000-1/280 (Greenberg 2001). This medical condition is the outcome of cerebrospinal fluid drainage obstruction that results in CSF accumulation and increased intracranial pressure (Bulat and Klarica, 2010), which in turns leads to neurological deficit, brain damage and death (Brodbelt and Stoodley 2007, Oreskovic and Klarica, 2011). The specific causes and sites of obstruction are currently not fully known (Johnston et al. 2004, Johanson et al. 2008), but two areas of the brain have been suggested to have important roles in the condition: the choroid plexus (site of CSF production), (Catala et al. 1998, Rodriguez et al. 2006, Zhao et al. 2011, Grapp et al. 2013) and the leptomeninge arachnoid (where CSF exits the brain), (Conegero and Chopard 2003, Cornelius et al. 2011).


Past research in this laboratory used the hydrocephalic Texas rat as the animal model to demonstrate that development of the fetal cerebral cortex is impaired in hydrocephalus through compositional changes in CSF (Masayekhi et al. 2002 and Owen-Lynch et al. 2003), and specifically through changes in folate and folate-associated proteins (Nabiuni et al. 2004). Moreover, a lack in CSF of a key folate enzyme: 10-formyl-tetrahydrofolate dehydrogenase (FDH) was associated with reduced cell proliferation and cortical plate thickness (Nabiuni et al. 2004 and Cains et al. 2009). Decreased FDH concentration in CSF meant the folate metabolic cycle was altered, rendering the brain deficient in specific folates. In this scenario, a folate treatment based on a mixture of two specific folates was devised, taking into account the metabolic reaction where FDH is involved. The treatment, administered through maternal supplementation, decreased the incidence of hydrocephalus

by 60%. Preliminary in vitro studies also showed that hydrocephalic fetal rats exhibited an elevated concentration of 5mTHF in CSF compared to Sprague Dawley (SD) control rats (Cain et al. 2009).

The role of FDH in the brain is yet not understood (Champion et al. 1994, Anguera et al.2006) as it is not the accepted mechanism for delivery of folates to specific areas like the cerebral cortex and meninge arachnoid (Rodriguez et al.2006, Grzybowski, et al. 2006, Antony and Heintz, 2007, Johanson et al 2008, Wollack et al 2008, Zhao et al. 2011, Grapp et al. 2013). In this context, this investigation aims firstly to elucidate the role of FDH in cell growth in the presence and absence of different types of folates, as well as to identify FDH's specific location, expression and potential interrelationships with known folate receptors (FRα) (Zhao et al. 2011 and Grapp et al 2013). for folate delivery to the developing brain. Past immunostaining analysis of the fetal brain located FDH on the fetal cerebral cortex of gestational day 20 H-Tx and SD control rats (Cains et al. 2009). In the current study, we used "in situ" PCR, and suggest that FDH is produced in the cerebral cortex, and that it binds to folates from the CSF at the level of the radial glia in the germinal epithelium. Folates bound to FDH are transported in the cerebral cortex by radial glia, as well as in the CSF, to the required areas for further development (Dziegielewska et al. 2000 and Miyan et al. 2001). The FDH-folate transport system may thus present an alternate folate transport mechanism to the classic folate transport by folate receptors and carriers (FRα, PCFT and RFC) in the choroid plexus (Wollack et al. 2008 and Steinfeld et al. 2009).

A second aspect of this research is related to the leptomeninge arachnoid. CSF flow in the brain is maintained thanks to a flow balance between CSF production in the choroid plexus and absorption in the arachnoid villi (Brown et al. 2004, Oreskovic and Klarica 2011). Studies by other research groups showed that impeded CSF absorption results in an altered flow and CSF accumulation leading to hydrocephalus (Grzybowski, et al. 2006, Glimcher et al. 2008 and Cornelius et al 2011). In addition, preliminary studies in our lab showed how hydrocephalic CSF has a strong inhibitory effect on cerebral cortex cell proliferation (Cains et al. 2009). We suggest that hydrocephalic CSF may also be inhibitory or even cytotoxic for the arachnoid, the main site for CSF egress resulting in drainage insufficiency. Consequently, the brain improvement observed in the offspring after our folate treatment was possibly due to increased CSF flow through the arachnoid villi, and we postulate that arachnoid functionality may be affected by FDH depletion in the hydrocephalic CSF, leading to reduced CSF outflow. Thus, our previous studies suggest that with folate supplementation, the hydrocephalic brain bypass the impaired FDH step in the folate cycle allowing in this way reestablishment of flow circulation and normalized cerebral cortex development at the same time (Cains et al. 2009). However, the folate mechanisms to explain these two parallel events are still poorly understood (Grapp et al. 2013), and in this context, the current studies focus on elucidating the role of FDH in folate transport in the presence and absence of folates, its interrelationship with folate receptors such as FRα (the main receptor involved in brain folate transport (Steinfeld et al. 2009 and Grapp et al. 2013), and the potential role of FDH and folates in cerebral cortex and arachnoid cell growth and differentiation. Finally, the present study suggests new avenues for further research into specific metabolic pathways potentially activated and inactivated in congenital hydrocephalus. To this end, we used for the first time a bioinformatic tool called Ingenuity Path Analysis (IPA), (www.ingenuty.force.com) to analyse metabolomics' data obtained from LCMS analysis of CSF in our previous studies.

## 1.2 CNS DEVELOPMENT AND CONGENITAL HYDROCEPHALUS

### 1.2.1 CNS development

The development of a complex structure such as the CNS requires the simultaneous and interdependent action of several developmental processes including morphogenesis, the establishment of positional identities and histogenesis. The individual regulation and coordination between these mechanisms is only partially known.

Cerebral cortex development in vertebrates starts at the expanded anterior end of the neural tube (see Figure 1.1 below). The notocord exerts its effects on the neural plate through secretion of sonic hedgehog (shh) (Echelard et al., 1993; Martı´ et al., 1995).
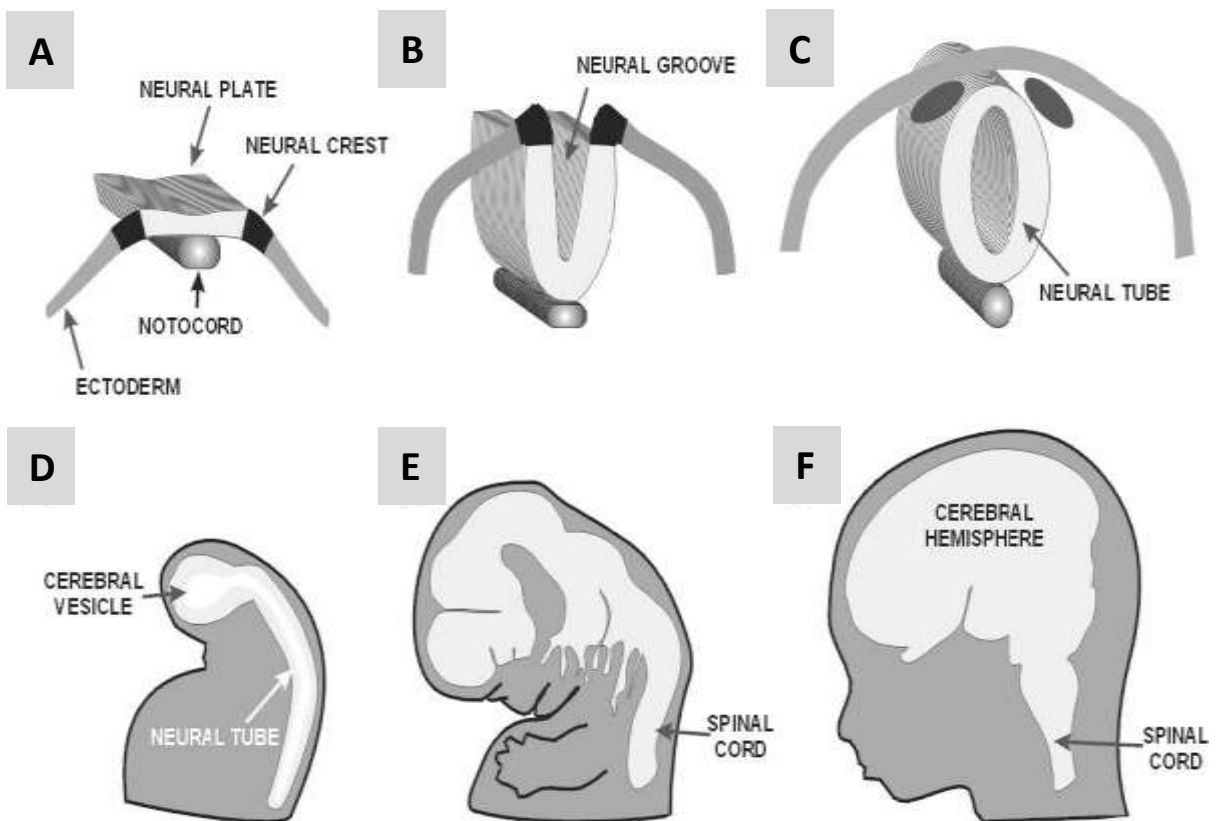
**Figure 1.1. Development of the CNS. Development of the CNS starts with proliferation of ectodermic cells that differentiate to give rise to the neural plate and neural crests (a). Neuroectoderm folds and gives rise to the neural groove (b). Neurolation is directed by an underlying notochord that secretes paracrine factors (d, e, f). The anterior end of the tube swells to form the cerebral vesicles that determine the shape of the brain including the cerebral hemispheres (f). (Miyan, J.A. et al. 2003)**

The brain and spinal cord walls are lined with a pseudostratified neuroepithelium containing neural stem cells with autonomous behaviour and rapid proliferation. Proliferation is stimulated and coordinated through the secretion of autocrine and paracrine molecules. (Panchision and McKay, 2002, Vaccarino et al. 1999)

Organizing centres have been identified within the neuroepithelium. These are the floor plate, the mesencephalic-rhomboencephalic isthmus, and the anterior part of the telencephalic vesicles as well as adjacent structures such as the notochord (Bueno et al., 1996; Crossley et al., 1996). The organizing centres yield diffusible molecules that are identifiable by cell receptors within the organizing centre or at a certain distance from it. These secreted molecules have an important role in the patterning or regionalisation of the fetal brain (Shamim et al., 1999; Vaccarino et al., 1999a, 1999b; Toresson et al., 2000; Garda et al., 2001; Panchision and McKay, 2002,).

In previous work in our laboratory, FDH in normal and abnormal brains was found to be located in the radial glia of the cerebral cortex and in CSF inside vesicles close to the germinal epithelium of the cerebral cortex, but not in other important areas of the brain where proteins are secreted into CSF i.e. choroid plexuses (Cain, 2009).

In the cerebral cortex, progenitor cells give rise to radial glia which shows interkinetic nuclear migration (see Figure 1.2, 1.3 and 1.4 below). The nucleus migrates to the inner ventricular surface just before mitosis and divides into two daughter cells, while the nuclei of the daughter cells move away from the inner ventricular surface during S-Phase to later move back to the ventricular surface to complete division (Norden et al. 2009). Progenitor cells are the precursors of all types of neurons and glia, except microglia in the cerebral cortex. Neurons and glia migrate away from the VZ to continue differentiating into mature

neurons, astroglia and oligodendrocytes. It has been suggested that progenitor cells move

to the ventricular surface to get access to the nutrients present in CSF, and that CSF acts a

growth medium and orchestrator of development (Miyan 2003).
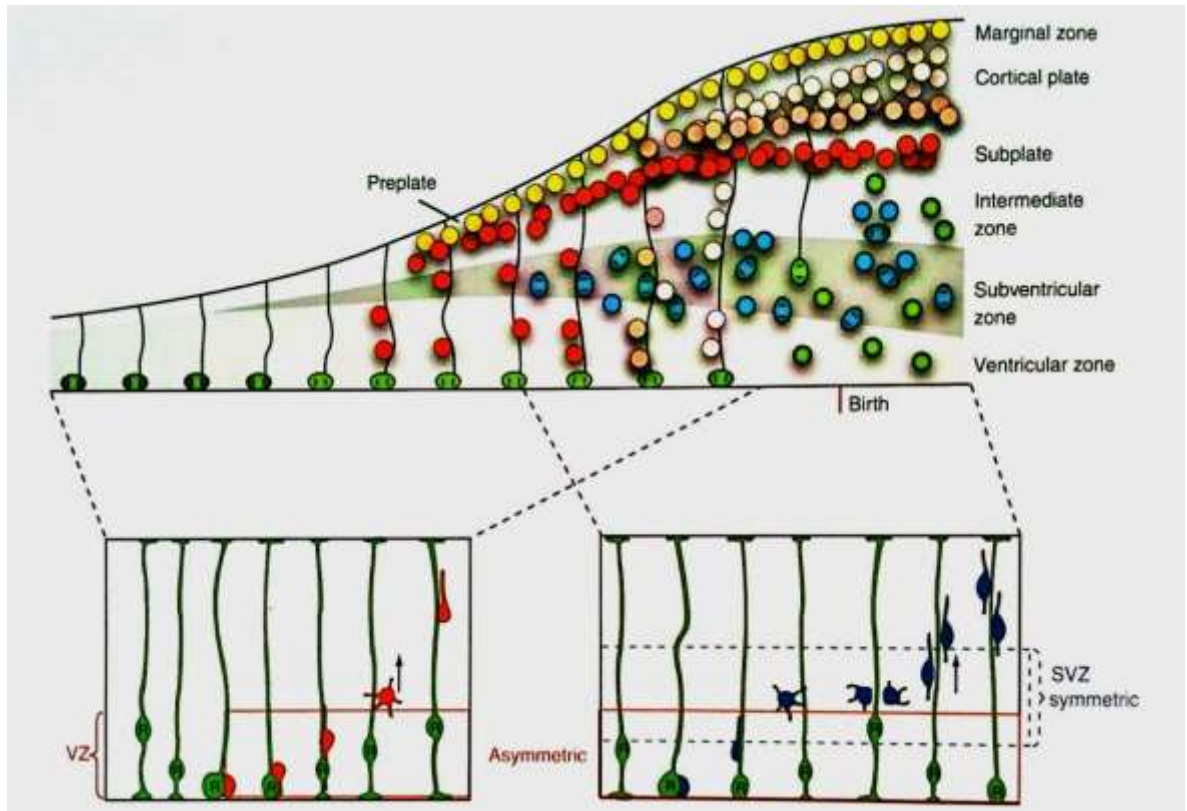


**Figure 1.2. Layers of the cerebral cortex. Six layers have been described in the cerebral cortex of mammals:  The ventricular zone (VZ), the subventricular zone (SVZ), the intermediate zone, the subplate, the cortical plate and the marginal zone. The cerebral cortex begins as a simple neuroepithelium made up of apical progenitor cells also called radial glia (green cells). As they proliferate they develop a bipolar shape. Radial glia divides symmetrically or asymmetrically and represents the scaffolding for neurons and astroglia that originate from them by mitotic division (Sanes, DH 2010).**
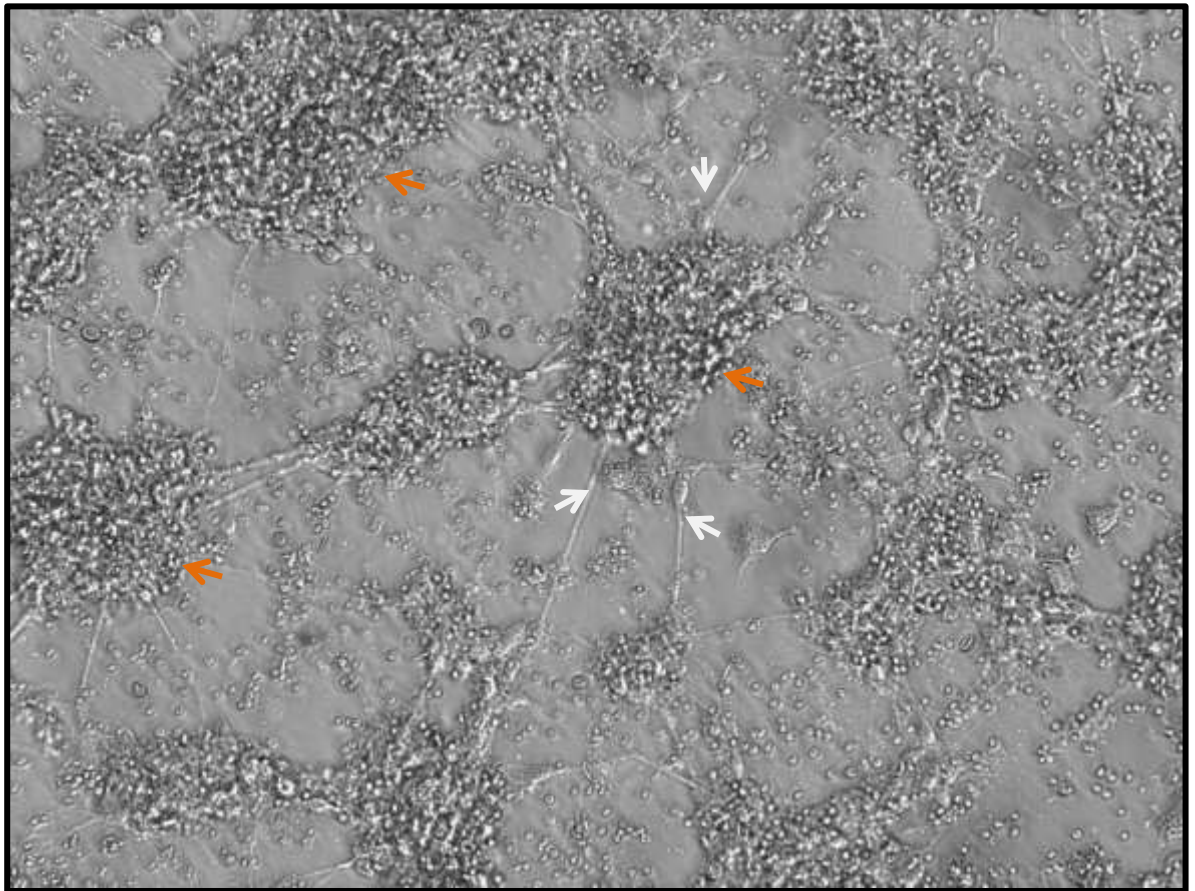
**Figure 1.3. Primary cell culture micrographs of cerebral cortex progenitor cells (orange arrows) showing cell projections and cell differentiation into radial glia (white arrows) at embryonic day 18. (Interference microscope, x20)**
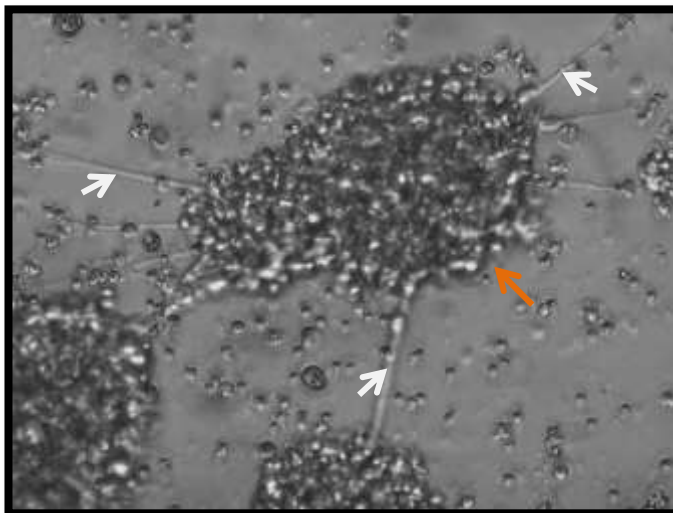


**Figure 1.4. Cerebral cortex progenitor cell (orange arrow) showing radial glia cells (white arrows) (Interference microscope x40).**

Migration is made possible by a group of specific hierarchical signals from the germinal epithelium to the cortical areas. The cells in the marginal zone (Cajal-Retzius) secrete the extracellular glycoprotein reelin that stimulates receptor and intracellular signalling systems across the cerebral cortex, and mediates migration and stratification (Cooper, J.A. 2008).

During development, the cavities of the brain ventricles are filled with protein-rich embryonic CSF. Although the role of this fluid has not been fully characterized, it is considered to directly influence neuroepithelial stem cell growth and proliferation, and differentiation of the cerebral cortex by means of growth factors FGF2 and EGF that are present in embryonic CSF (Panchision and McKay, 2002; Rajan et al. 2003). Furthermore, some studies have found that the protein content in CSF is more complex in embryos than in adults. A greater protein concentration in embryonic CSF suggests that it has a role in the regulation of cerebral cortex neuroepithelial cell behaviour (Dziegielewska et al., 1980b, Dziegielewska et al., 2000, Ojeda and Piedra, 2000; Miyan et al., 2003; Gato et al., 2004). Embryonic CSF also plays a key role in the expansion of the brain at the earliest stages of growth (Desmond and Jacobson, 1977; Desmond, 1985; Alonso et al., 1998), and abnormal embryonic CSF in some congenital malformations has been associated with altered cortical brain development (Mashayekhi et al., 2002; Owen-Lynch et al., 2003). Gato and colleagues (2005) studied the effect of embryonic CSF on mesencephalic neuroepithelium, and revealed its inability to self-stimulate basic cellular behaviour "in vitro", concluding that normal behaviour is promoted by extraneural signals and diffusible factors present in embryonic CSF. In conclusion, the aforementioned body of work highlights the importance of embryonic CSF in fetal brain development. Notably, this work is also in accordance with our results from CSF LCMS analysis of Texas rats. At embryonic day 18, CSF LCMS analysis showed a 6-fold FDH decrease in CSF compared

to embryonic day 16, when the closure of the neural tube in both normal unaffected and affected Texas rats is still incomplete and CSF flow is still restricted due to an as yet unopened SAS. Therefore, it was proposed that the reduced FDH levels observed in CSF at later embryonic days (day 18) were a response to the high demand for this enzyme and associated folates at earlier stages of development (day 16), when FDH is needed for completion of the neural tube closure as well as for the establishment of CSF flow circulation and drainage (Dr. Miyan personal communication).

**1.2.2 Congenital hydrocephalus**



Normal fetal brain
with ventricles
containing CSF

Hydrocephalic fetal
brain with enlarged
ventricles caused by
CSF obstruction and
fluid built-up

Spina bifida
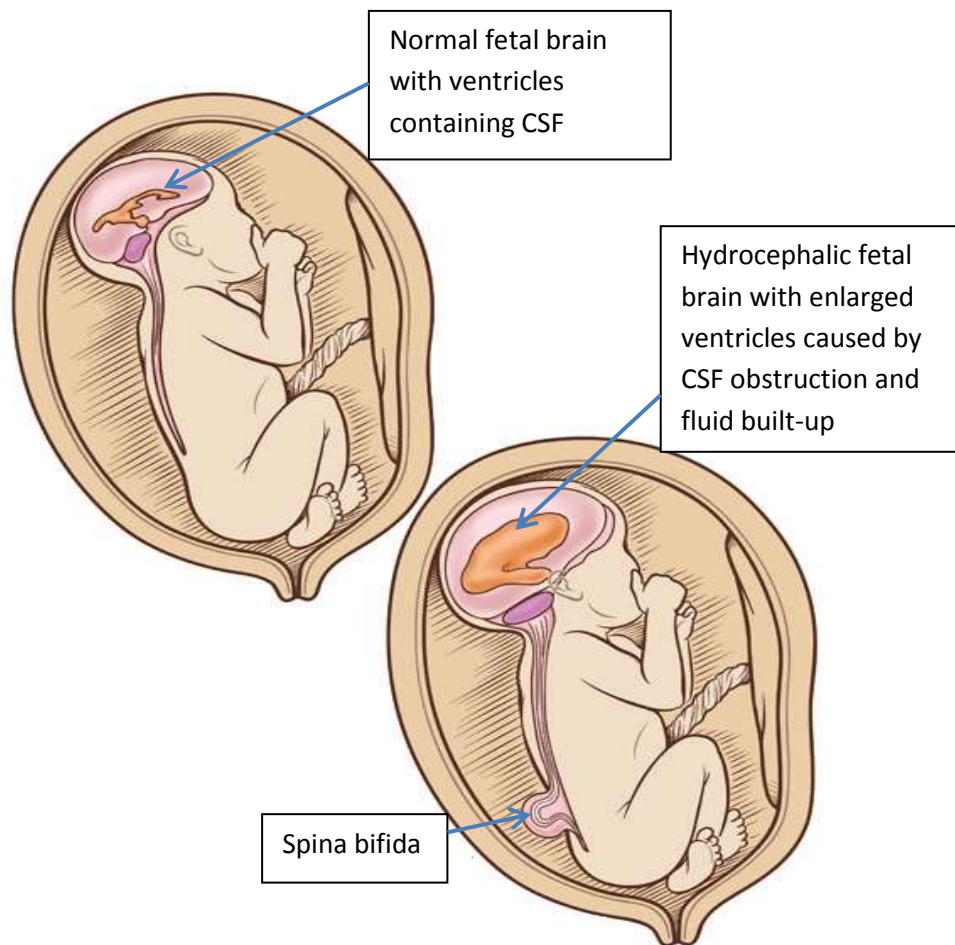
**Figure 1.5. Schematic representation of the normal and hydrocephalic fetal brain. The image above shows a normal fetus surrounded by placenta and normal size brain ventricles. The image below represents a hydrocephalic brain with enlarged ventricles and spina bifida, a condition also associated with congenital hydrocephalus. Adapted from www.mottchildren.org**

Congenital hydrocephalus is considered to be caused by accumulation of CSF in the fetal brain due to obstruction of the common CSF pathways leading to ventricular distension and brain injury (Oreskovic et al. 2010 and Miyan et al. 2003). This condition affects from 1 in 500 to 1 in 5000 children worldwide (Schurr et al. 1953, Greenberg et al. 2001, Sipek et al.2002 and NIH 2005) and it occurs in isolation or in association with other disorders such as neural tube defects, spina bifida (figure 1.5) and Chiari II syndrome (Liddon 2004).

Due to the broad variety of events leading to this condition, congenital hydrocephalus is one of the most challenging pediatric diseases in terms of medical complications. It is known that fetal-onset hydrocephalus is triggered by an alteration in CSF dynamics provoked by fluid drainage insufficiency or obstruction. This leads to fluid accumulation in the brain ventricles, as well as raised intracranial pressure and ventricle enlargement; the latter is the main phenotypical characteristic observed in fetal brain scans (Del Bigio et al. 2010). However, congenital hydrocephalus should not be seen only as a mechanical phenomenon, but rather a sequence of disorders occurring at a specific time and brain location (Rodriguez et al. 2006). Although the pathophysiology of congenital hydrocephalus is still not fully understood, despite more than 100 years of research, extensive literature has reported numerous brain alterations associated with this disorder. These alterations include brain loss in the form of severe reduction in cerebral cortex thickness, and abnormal development of certain neuron sub-types, resulting in cognitive deficits (Cain et al. 2009, Miyan et al. 2003, and Rodriguez et al. 2006). Brain loss is also a consequence of neuroepithelium denudation, which leads to obliteration of the Sylvius Aqueduct and CSF accumulation as well as alterations of the hypothalamus and neuroendocrine deficiencies (Mashayekhi et al. 2002, Rodriguez et al. 2006 and Johanson et al. 2008). Nevertheless, congenital hydrocephalus is also accompanied by brain gain, in terms of elevated astrocyte proliferation around the Aqueduct of Sylvius and third ventricle, in response to brain loss and an attempt to restore brain areas negatively affected by denudation (Rodriguez et al. 2006).

CSF accumulation in the fetal brain is also linked to intraventricular haemorrhage (IVH) at a time in development (24 to 32 weeks' gestation) when the subependymal germinal matrix in brain ventricles is metabolically highly active. Further, it is in contact with

capillaries not supported by adventitia and unprotected in terms of autoregulation, making the fetus vulnerable to brain hemorrhage (Cherian et al. 2004). 25% of low birth weight babies develop posthemorrhagic hydrocephalus (PHH) (Batton et al. 1994 and Sheth et al. 1998), and neurological deficiencies are more accentuated in PHH than IVH (Murphy et al. 2002 and Sasidharan et al. 1986). The specific causes for PHH and IVH are unknown; however, it is thought that hemorrhage may occur after blockage of CSF by various mechanisms including CSF obstruction by blood products, inflammation in brain sites closed to CSF pathways, gliosis and arachnoidal inflammation. Furthermore, ventricular distension and therefore physical stress leads to white matter injury in PHH and IVH (Volpe et al. 2008 and Du Plessis et al. 1998). It has been suggested that brain injury in babies with IVH and PHH may also be due to cytotoxins from blood products that damage the germinal matrix of the ependyma after hemorrhage (Du Plessis et al. 1998).

There is molecular genetic evidence that in humans, 5 to 15% of cases of congenital hydrocephalus are caused by missense mutations in the X sexual chromosomes. The mutations are distributed specifically within the Xq28 gene encoding for the functional domains of L1CAM protein or L1 protein, an immunoglobulin involved in neural cell adhesion in neurons and Schwann cells (Jouet et al. 1993). X-linked recessive inheritance in females means they are carriers of the condition, whereas males with the mutant recessive gene die after birth (Renier et al. 1982). In contrast to humans, where only one gene has been associated with congenital hydrocephalus (Xq28), investigations in animal models found that 9 genes and more than 43 mutants/loci are linked to hereditary hydrocephalus, with the products of these genes being mainly cytokines, growth factors and molecules related to cellular signal pathways activated during early brain development (Zhang et al. 2006). Unfortunately, there is very limited understanding about the genetic

and molecular mechanisms involved in human hydrocephalus and for this reason it is not yet possible to extrapolate to humans the genetic data obtained from animal models.

## 1.3 CSF PRODUCTION AND FLOW CIRCULATION

### 1.3.1 CSF production.

CSF is produced in the ventricular system by highly vascularized small organs called choroid plexuses (Figure 1.6 and 1.7). They start developing between the second and third month of pregnancy in humans and originate from the neural tube after its closure, arising from an extension of the ependyma lining the lateral, third and fourth ventricles (Hartwig and Paulus, 2010, Gato et al. 2004, Catala et al., 1998 and Dziegielewska et al. 2001).

The choroid plexus has morphological and functional polarity presenting ciliated, cuboidal choroidal cells, which are joined by tight junctions at the apical side. These cells form the apical membrane which is in contact with CSF, and on the opposite site a basal membrane with lateral interdigitations in immediate contact with fenestrated (leaky) nearby cerebral capillaries (Figure 1.7). The endothelium of capillaries, basal and apical membranes, and CSF form the blood-CNS barrier. The adequate functioning of this barrier is critical for CSF production and regulation of the brain's internal environment through production and movement of solutes that help to maintain homeostasis, CSF hydrodynamics and general brain health (Johanson et al. 2008, Hartwig and Wernes 2010, Oreskovic et al. 2010).

Human CSF is produced at a high rate (0.5 ml/min/g tissue) which varies from 0.3 to 0.6 ml/min. Production is controlled by hormonal and neuroendocrine modulation, and most CSF is contained in ventricles (25% of total space) and subarachnoid space (75% of total space) (Johanson et al. 2008). With age and in neurodegenerative diseases like dementia and Alzheimer, CSF volume increases while CSF outflow decreases leading to accumulation of toxic substances within the brain (Rubenstein 1998, Silverberg et al. 2003)

At early stages of fetal brain development, there are unique barrier mechanisms between blood, CSF and brain. These cause elevated levels of solutes (proteins) in the CSF by allowing high rates of transport of specific proteins from blood to CSF through the tubulocisternal endoplasmic reticulum of choroidal cells (Dziegielewska et al. 2000). Interestingly, these proteins are prevented from entering the brain extracellular space thanks to barriers at the CSF-brain interfaces on the ventricular lumen and the Piamater. These barriers consist of junctions between choroidal cells and disappear at early stages when CSF decreases its protein content in adulthood (Dziegielewska et al. 2000). The presence of unique barriers at early stages is crucial for prevention of free exchange of molecules at a critical time for the fetal brain, when CSF transports important signaling molecules for coordination of brain development (Dziegielewska et al. 2000 and Miyan et al. 2003). In addition, high protein content in CSF at early stages also favours the production of an elevated osmotic pressure in the ventricular system which contributes to ventricle expansion as the brain grows (Dziegielewska et al. 2000 and Dziegielewska et al. 2001). Since the protein content in CSF is much lower than in serum, the blood-CSF barrier permeability in the immature brain decreases with time and eventually becomes highly efficient at impeding movement of potentially damaging molecules from plasma to CSF through active transport mechanisms in the mature brain (Dziegielewska et al. 2000, Zhao et al. 2011).

The choroid plexus is a kidney-type organ and it has similar renal epithelial mechanisms and transfers a large volume of ions and water (Johanson et al. 2008). The secretion of ions, water and proteins for CSF production is an active process that involves pumps, ion channels, cotransporters and antiporters, and aquaporins that control fluid and solute exchange (Redzic and Segal 2004). Movement of solutes by membrane transporters occurs

in the basal membrane (plasma-facing) and apical membrane (CSF-facing) of the choroid plexuses, inducing osmosis as well as the transport of water and molecules inside the choroid plexus (Kimelberg 2004, Speake et al. 2001, Brown et al. 2004, Emerich et al. 2005).

Transport mechanisms in the choroid plexus respond to CSF variation in composition and concentration by modulating the transport of solutes through membrane receptors. Receptors are stimulated by CSF neuropeptides to alter metabolism of secondary messengers and the ion transport occurring in parallel with CSF production (Scavone et al. 1995). Hormones like vasopressin and angiotensin II stimulate their corresponding receptors and induce choroidal cells to down-regulate CSF production (Johanson et al. 1999). Similarly, stimulation of vasopressinergic receptors by hormones results in reduction of Cl$^-$ efflux from choroid plexus epithelium and decreased CSF formation (Faraci et al. 1990). Atrial natriuretic peptide (ANP), a regulator of AQP-1 channels, is also involved in modulation of CSF production through downregulation or upregulation of receptors' expression in hydrocephalus (Steardo et al 1987). Variations in ANP receptor density and CSF production also follow changes in CSF hydrodynamics (Mori et al. 1990). Transport of proteins and vitamins across the choroid plexus also occurs through endocytosis and transcytosis (Smith et al. 2004, Zhao et al. 2011). Vitamin transport within the choroid plexus, specifically, is mediated by vitamin receptors and carriers, and this is described in detail in figures 1.14 page 58. A hypothetical model for folate transport from the choroid plexus to cerebral cortex and possibly to other areas of the brain by means of exosomes acting as folate shuttles in CSF was recently reported by Grapp et al. in 2013 and this model is described in figure 1.15 on page 61.

In conclusion, the choroid plexus has a secretory function in the release of water, proteins, ions, vitamins, hormones and growth factors into CSF. Interestingly, this function happens in parallel to a second function, which is the clearing of CSF acting as "ectopic renal tubular epithelium", removing toxic metabolites from the fluid by pumping them into the blood, "filtering" CSF in reverse direction to the known blood filtration system observed in the kidney (Johanson et al. 1995 and Johanson et al. 2008).
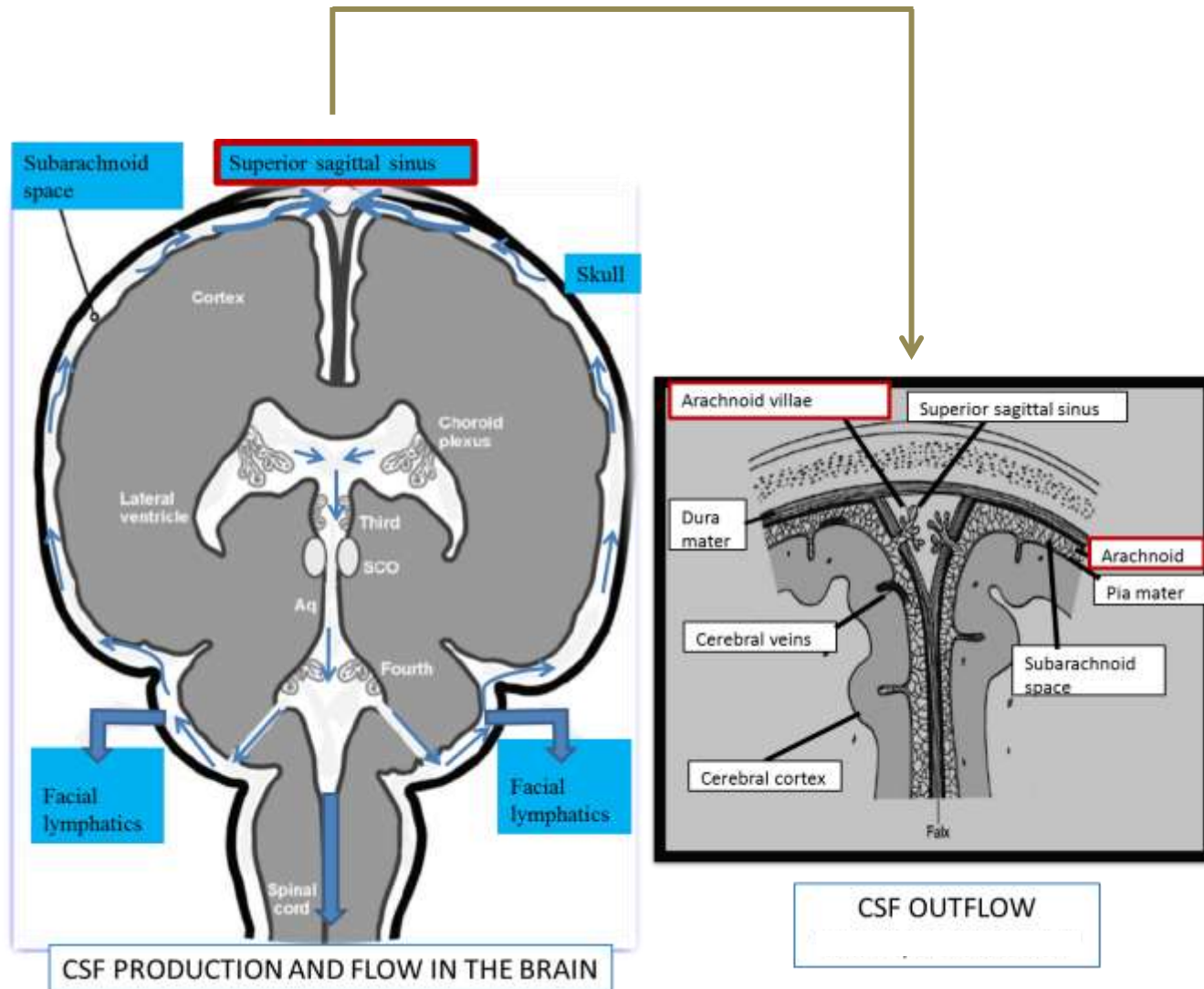
**Figure 1.6. CSF FLOW. CSF (blue arrows) is produced in the choroid plexuses of lateral, third and fourth ventricles and progresses towards the spinal cord, facial lymphatics and the subarachnoid space, leaving the brain through the arachnoid villae in contact with the superior sagittal sinus. The grey arrow indicates the CSF outflow area (arachnoid and superior sagittal sinus), (square).**
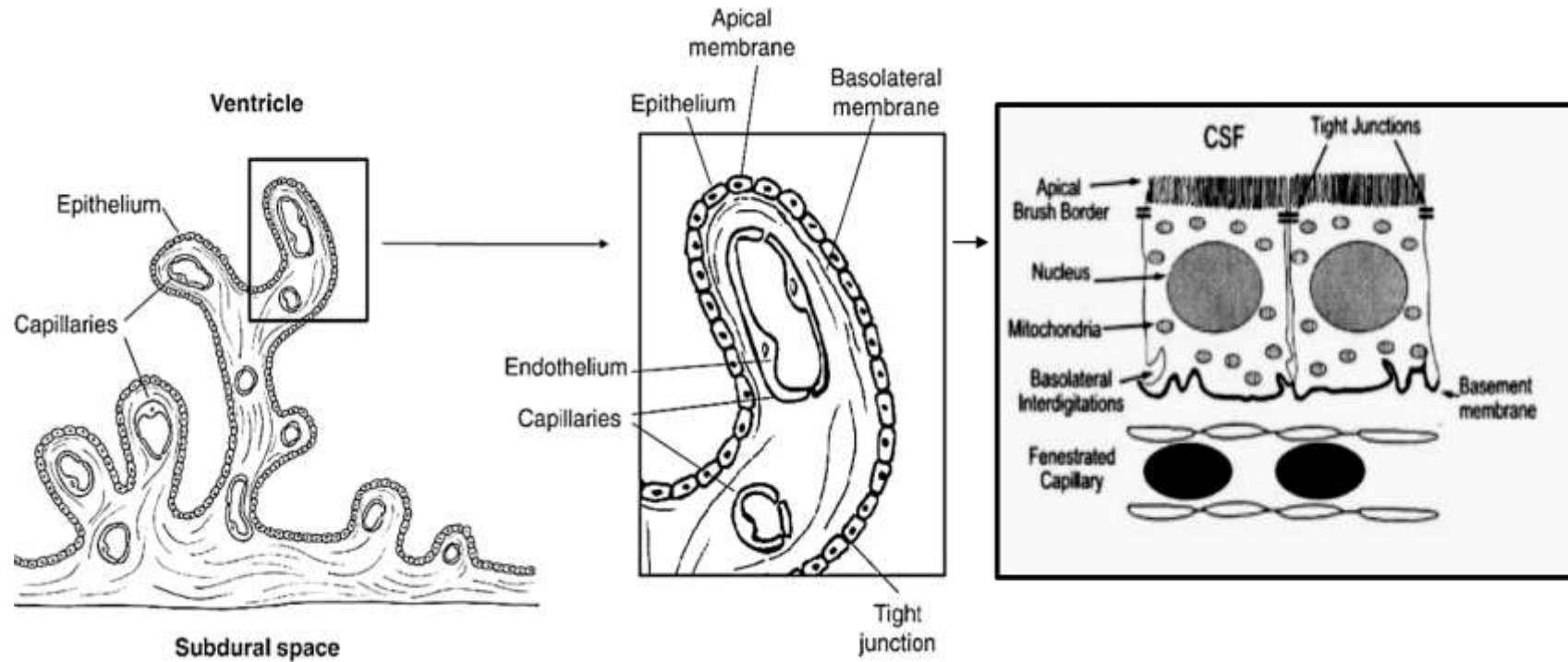
**Figure 1.7. Choroid plexus structure and the blood-CSF barrier. The choroid plexus is formed of ciliated and cubical epithelia cells joined by tight junctions at the apical membrane bathed by CSF. At the opposite site of the apical membrane, the basal membrane is in contact with fenestrated capillary. Capillary, basal membrane and apical membrane along with the CSF form the blood-CSF barrier. Numerous unique fenestrated capillaries go through the choroid plexus and connect creating an intricate network (Speake, T. et al. 2001). The choroid plexus epithelia folds, creating branched structures which contain a large number of villi. (Modified from Oreskovic et al., 2010 and Johanson et al. 2008)**

### 1.3.2 CSF flow circulation

According to the traditional hypothesis of hydrodynamics, CSF circulation is possible thanks to the cardiac cycle, which creates a pulsatile flow from the main site of production (choroid plexuses) to the sites of absorption (arachnoid villi or granular arachnoid and nasal facial lymphatics) (Wagshul et al. 2011) (see Figure 1.6) It is also thought that CSF pulsations depend on arterial hemodynamics in the choroid plexuses (Egnor et al 2002). CSF flow is unidirectional and fluid is actively and continuously produced against intracranial pressure. Formation of CSF and reabsorption is balanced to keep high CSF pressure within then ventricles (100 mm $H_2O$) in humans (Upton et al. 1985). At the same time, CSF pressure within the ventricles is higher than vascular pressure in dural venous sinuses, and disturbances of CSF pressure (low pressure) by toxic substances can inhibit production of fluid by the choroid plexus (Murphy et al. 1989, Johanson et al. 1990 and Maren 1988). CSF flows from the lateral ventricle to the III and from the III to the IV through the aqueduct of Sylvius (Figure 1.6). The flow continues from the IV ventricle, through Foramina into the subarachnoid space, and finally reaches the granular arachnoids where it is "filtered" and passed to the superior cortical sagittal sinuses in the subarachnoid space and the spinal subarachnoid spaces (Egnor et al. 2002). Most of the CSF leaves the brain through the leptomeninge arachnoid, and a small proportion is absorbed in the facial lymphatics (Miyan et al. 2003). CSF is constantly replenished with an average production of 500ml in 24 hours, and it is the continuous CSF secretion into the ventricles wthat creates a flow that allows CSF nutrients to reach every single area of the brain for cell division and cell differentiation into specialized tissues and organs of the CNS (Johanson et al. 1995 and Johanson et al. 2008). Only a balance between absorption and production of CSF can lead to permanent and normal fluid flow through the brain and subarachnoid spaces, and thus the correct functioning of the brain (Rekate, 2008).

## 1.4 CSF COMPOSITION

Adult CSF is colourless and very similar in composition to plasma but with lower protein content: 25 nM compared to 6500 nM in plasma (Segal 2001). 99% of adult CSF is water, but importantly, the remaining 1% comprises ions ($Na^+$, $K^+$, $Mg^{2+}$, $Cl^-$ and $HCO_3^-$) vitamins (folates), hormones (angiotensin and vasopressin), growth factors and proteins principally globulins, albumin and immunoglobulins which are crucial to provide an optimal microenvironment for brain cell function (Segal 2001, Chodobski and Szmydynger-Chodobska, 2001). On the other hand, CSF in the developing brain, particularly in the fetal stages, is more complex and is highly rich in protein content, acting as a growth medium for neural stem cells, and providing all the components needed for neuroepithelial cell proliferation and cell survival, neurogenesis and CNS development (Dziegielewska et al 2000, Gato et al. 2004, Bueno et al. 1996, Miyan et al. 2003). The elevated protein content in fetal CSF is due to unique barrier mechanisms between blood, CSF and brain that encourage transport of specific proteins from blood to CSF through the tubulocisternal endoplasmic reticulum of choroidal cells (Dziegielewska et al. 2000). High protein content in CSF at early stages favors the production of an elevated osmotic pressure in the ventricular system, which contributes to ventricle expansion as the brain grows (Dziegielewska et al. 2000 and Dziegielewska et al. 2001). Remarkable evidence about the elevated level of proteins in embryonic CSF compared to adult CSF was found by Gato and colleagues in 2004, who demonstrated that at early stages of a chick's brain development there is a progressive accumulation of proteins in CSF.

Proteins in fetal CSF need to be prevented from entering the brain's extracellular space. Otherwise, brain functioning can be compromised, and it is thanks to barriers at the CSF-

brain interfaces on the ventricular lumen and the Piamater that free exchange of molecules is avoided at a critical time when CSF transports key signaling molecules for coordination of development in the developing brain (Dziegielewska et al. 2000 and Dziegielewska et al. 2001, Miyan et al. 2003). Also, since the protein content in fetal CSF is much lower than in fetal serum, the blood-CSF barrier permeability decreases with time and eventually becomes highly efficient in impeding movement of potential damaging molecules from plasma to CSF through active transport mechanisms (Dziegielewska et al. 2000, Johanson et al 2008, Gato et al. 2004, Zhao et al. 2011).

The key components in CSF, other than proteins, are ions. These are present in adult and fetal CSF, and are involved in osmosis, a process coupled with the transport of water inside the choroid plexus for CSF production. These ions are sodium ($Na^+$), chloride ($Cl^-$), and bicarbonate ($HCO_3^-$). $Na^+$ efflux from choroid plexus to CSF is controlled by Na+/$K^+$ ATPase pumps that maintain the adequate $Na^+$ concentrations inside and outside the choroidal cells, and create an osmotic gradient through the apical membrane to keep $Na^+$ intracellularly low. Low intracellular $Na^+$ also encourages the transport of $Na^+$, present in blood, through the choroidal basal membrane by means of $Na^+/H^+$ and $Na^+/Cl^-$ co-transport (Figure 1.8). This continuous $Na^+$ influx through the basal membrane into the choroidal cells, and the intracellular $Na^+$ efflux into CSF through the apical membrane results in a constant transport of $Na^+$ from blood to CSF and the creation of a $Na^+$ osmotic gradient that allows $H_2O$ to be transported from blood to CSF, (Brown et al. 2004). Additionally, water is secreted from the choroid plexus into CSF by means of trans-membrane channels called Aquaporins (AQP) which are present in the apical membrane (Figure 8). Inhibition of AQP1 has been suggested as a potential treatment for hydrocephalus (water accumulation in the brain), (Gunnarson et al. 2004). Other secondary active transport

involved in maintaining the osmotic gradient for CSF production occurs in the blood facing choroidal membrane to permit Cl⁻ inward transport through exchange with extracellular $HCO_3$. Intracelullar $HCO_3$ is also produced by carbonic anhydrase (c.a) from $CO_2$ for exchange with Cl⁻. K/Cl cotransport helps maintain cell volume (Johanson et al. 2008).
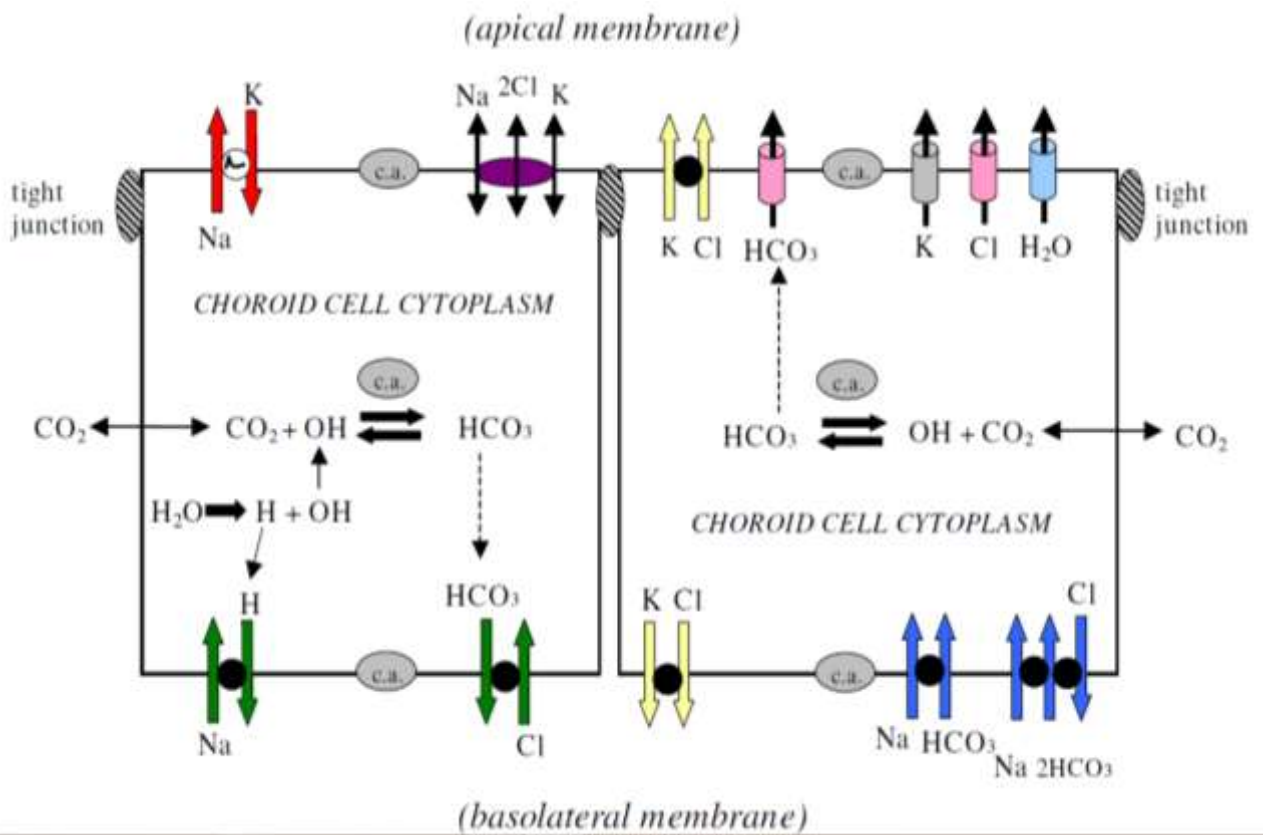


**Figure 1.8. Ion transporters and channels in mammalian choroid plexus. CSF production is possible thanks to coordinated transport of ions and water from the basolateral membrane to cytoplasm and from cytoplasm across the apical membrane into ventricles (Adapted from Johanson et al. 2008).**

CSF composition changes as it flows away from the site of production (choroid plexuses) to the site of absorption (arachnoid villae and nasal facial lymphatics). This is due to ion-exchange of CSF with the interstitial fluid that is produced from the capillary bed in the brain, and the ion-exchanged between CSF and the limiting membrane of neurones, piamater and glial cells (Johansson 1995). Additional components are added to the fluid from the choroid plexus, located in the third and fourth ventricles, as well as from the circumventricular organs circling the cerebral aqueduct at the exit of the third ventricle and the pineal gland (Miyan et al. 2003). For instance, the subcomissural organ (SCO) in the roof of the aqueduct of Sylvius (figure 1.6) secretes glycoproteins into CSF, with a function in axonal guidance and activity on neurite outgrowth. These glycoproteins produced early in development are called SCO-spondins and they aggregate in CSF to create Reissner's fibres forming thread-like structures that are part of the central canal of the spinal cord (Gobron et al. 2000 and Vio et al. 2008).

Abnormalities in CSF composition separately and/or in combination with impaired secretion, circulation and absorption have all been linked to different neurological conditions. For instance, high levels of immunoglobulins in CSF are indicative of inflammatory neurological disease (Harrington and Merril 1988, Chu et al. 1983). Alterations in the levels of nerve growth factor (NGF), transforming growth factor (TGF) and fibroblast growth factor (FGF) are associated with Alzheimer's disease (AD), (Nishio et al. 1998). Furthermore, NGF has been acknowledged as a biomarker for the diagnosis of multiple sclerosis, where this growth factor is highly increased (Suzaki et al. 1997). Other growth factors like stem cell growth factor (SCF) are thought to be linked to deficiencies in hematopoietic brain support caused by growth factor depletion in CSF and plasma, leading to AD (Laske et al. 2008). Our past investigations also demonstrated compositional

changes related to low levels of folate enzyme (FDH) in hydrocephalic CSF, which suggests that CSF variations in protein profile may lead to congenital hydrocephalus (Miyan et al. 2003, Cain et al 2009).

## 1.5 CSF ABSORPTION: THE LEPTOMENINGE ARACHNOID

### 1.5.1 Morphology

The leptomeninge arachnoid derives from the neuroectoderm, extending from the filum terminale to the external surface of the cerebrum, and is located between the meninges duramater and piamater (Figure 1.9).
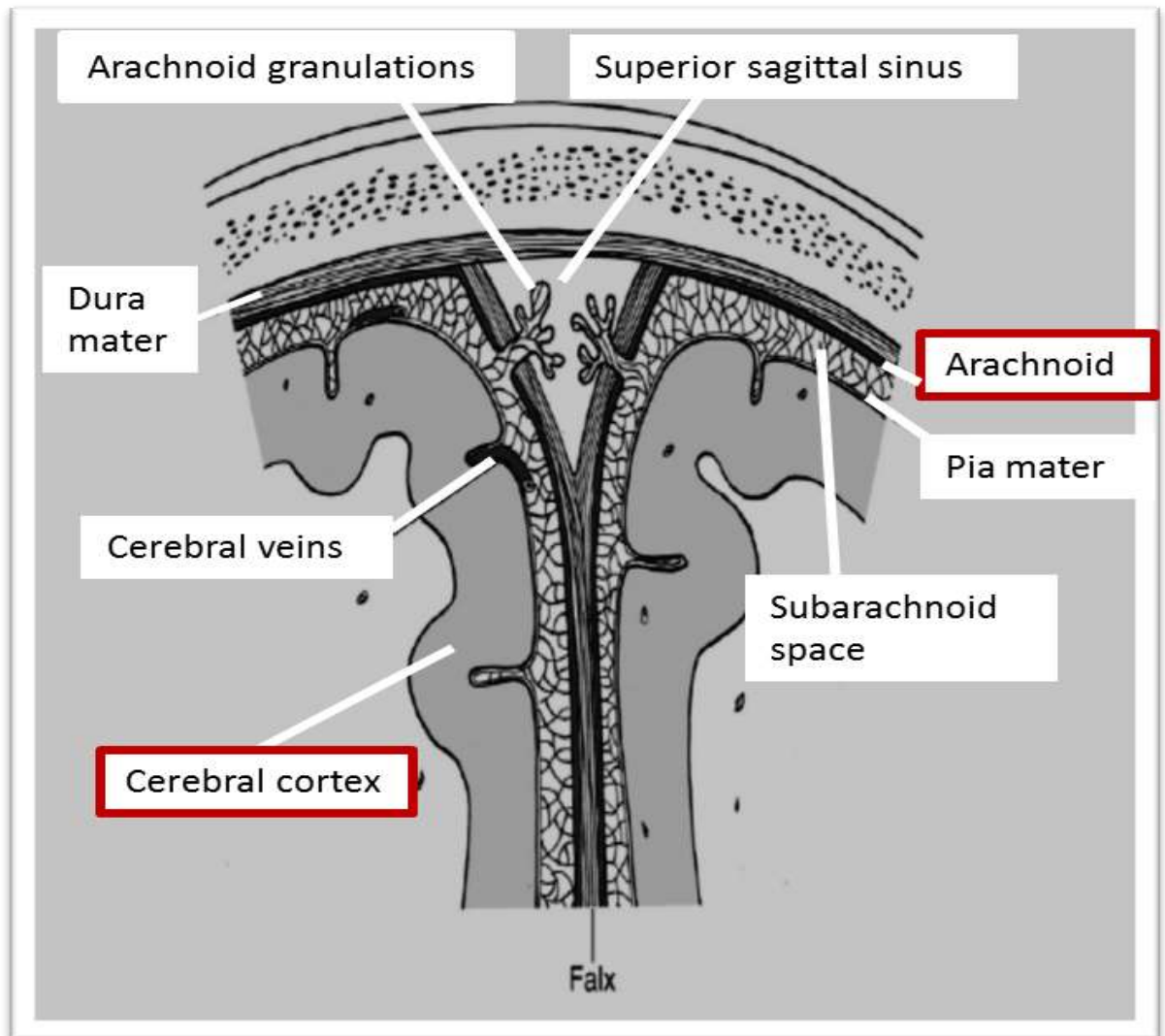


**Figure 1.9. CSF outflow through arachnoid granulations. The arachnoid is a web-like layer of the brain in contact with the duramater in its apical side and with piamater in the basal side. The arachnoid tissue protrudes to produce arachnoid granulations that allow an increased surface area for CSF drainage. Arachnoid granulations are in contact with the endothelia of the superior sagittal sinus for CSF egress (adapted from Oreskovic et al., 2010).**

Similarly to the choroid plexuses, arachnoid cells are part of the CSF-blood barrier, and have apical-basal polarity, forming tight junctions to create a barrier to solute and water flow (Janson et al. 2011). In the human fetal brain the arachnoid protrudes, forming the arachnoid microvilli that differentiate into arachnoid granulations at 18 months of age (Clark 1920). Arachnoid granulations are in contact with the dural wall of the sinus for CSF egress in conditions of high CSF pressure (Turner et al. 1961, Upton et al. 1985 and Zakharov et al. 2004). Arachnoid villi and granulations are similar in structure. Thus, the term 'granulation' is used to denote structures visible to the eye while "villi" are structures very similar in morphology but only observed with the aid of a microscope. Arachnoid granulations or arachnoid villi physiological role in the brain is still a subject of controversy and after several attempts to elucidate its involvement in CSF outflow the specific mechanism is not yet understood and awaits fully characterization.

Both, arachnoid villi and arachnoid granulation are composed of protuberances with small channels or tubules that remained closed within the venous sinus at low pressure and open when CSF pressure is high, working similarly to a one-valve mechanism (Welch & Friedman 1960, Upton et al. 1985 and Upton et al. 2010).
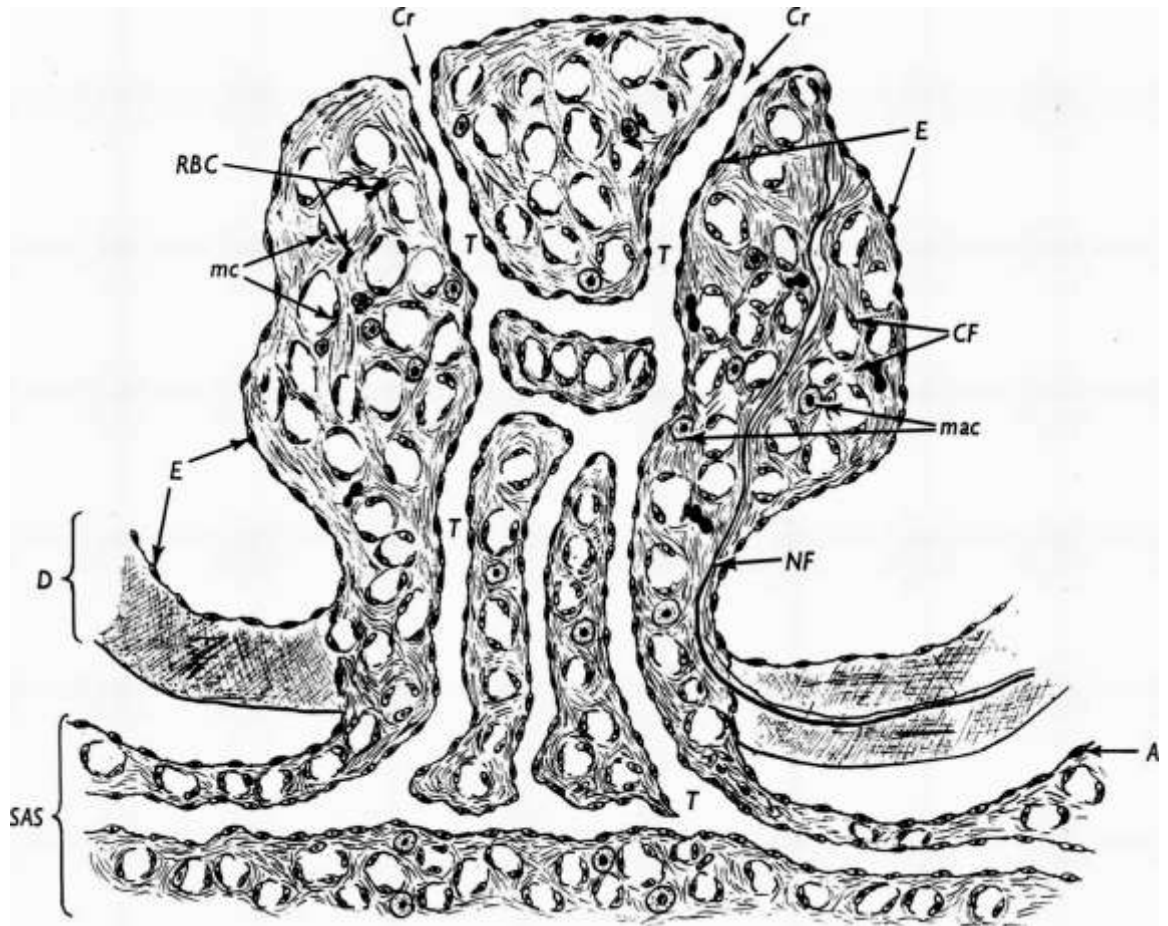
**Figure 1.10. Graphic reconstruction of mammal arachnoid granulation. SAS: subarachnoid space, D: dura, A: arachnoid membrane, T: tubule, NF: nerve fibres, E: endothelia, mc: mesothelial cell, mac: macrophage cell, CF: collagen fibres, NF: nerve fibres, Cr: crypt (adapted from Jayatilaka, 1965).**

Externally, arachnoid granulations are cauliflower-like shape structures with 1100 μ width and 1000 μ height that appear near the opening of cerebral veins into the superior sagittal sinus. Subarachnoid tissue and arachnoid membrane is compressed and through gaps in the dura reaches the venous sinuses to form part of their walls. Further, surface epithelial cells cover the arachnoid granulation to become part of the walls of blood vessels (endothelia) in the dura. Internally, arachnoid granulation presents crypts and channels or tubules lined with endothelia as well as a core of mesothelial cells that create networks (trabeculae) surrounded by collagen fibres that are at the same time engulfed by endothelia (Jayatilaka 1965, Conegero & Chopard 2003 and Grzybowsky et al.2006).

**1.5.2 CSF absorption**

A large body of research on CSF outflow models describes the arachnoid villae and arachnoid granulations as the main brain structures for CSF egress, with unidirectional flow through each single villus in situations of elevated CSF pressure, and lower surrounding blood pressure (Clark and Le gross in 1920, Bradbury 1979, Levine et al. 1982, Butler et al. 1984, Upton et al. 1985, Glimcher et al. 2008 and Holman et al. 2010). Arachnoid granulations are created as a result of raised CSF pressure, which forces the arachnoid villi to protrude further into the sinus. Such a phenomenon was observed by Butler in 1984 who detected numerous pinocytic vesicles in the endothelium lining the arachnoid villi. In another similar study, vacuoles, rather than vesicles were observed in the monkey sinus endothelium at elevated CSF pressure, suggesting that a macrovacuolar system may allow passage of particulate matter through arachnoid villi into the venous sinus and then into the general circulation (Tripathi et al. 1974 and Takahashi et al. 1993) Micropinocytotic vesicles, as well as paracellular transport in wider spaces between endothelial cells and large intracellular vacuoles, were also identified in human arachnoid granulation cells grown "in vitro" on filter membranes that were subjected to water flow pressure, mimicking the effect of CSF pressure on arachnoid granulation. These vacuoles fused to create transendothelial channels for transport of molecules from arachnoid granulation into venous sinus (Grzybowski et al. 2006). Other studies by Gupta and colleagues in 2010 revealed that CSF pressure is approximately 3 to 5 mmHg higher in the venous sinuses than in the granular arachnoid, and that this difference in pressure forces CSF flow from the subarachnoid space across the basal membrane of arachnoid granulations to the apical membrane, to finally drain into the venous sinuses. This finding supports previous published research and thoughts by many scientists, among others Tripathi et al. 1974, Upton et al.1985, Takahashi et al. 1993, Glimcher et al. 2008, Holman

et al. 2010 and Gupta et al. 2010). Thus, arachnoidal CSF drainage is considered the main approach to CSF outflow (Holman et al. 2010); however, how this occurs is not yet fully understood, and until now, the given explanatory mechanisms have remained controversial. In this context, other plausible sites proposed for CSF absorption are the olfactory nerves (Erlich et al. 1986) and optic nerves (Erlich et al. 1989) as well as the cribriform plate, nasal submucosa and cervical lymphatics (Mollanji et al. 2001, Koh et al. 2006, Johnston et al. 2004 and Johnston et al. 2005). An important alternative explanation for CSF outflow mechanisms is the work of Mollanji and colleagues in 2001. They demonstrated that obstruction of the cribriform plate impedes CSF drainage and increases intracranial pressure. Also in agreement with this investigation, Johnston and colleagues microfilm injection studies on non-human primates provided evidence that the main route for CSF drainage at low intracranial pressure is via the extracranial nasal lymphatics. These researches proposed that arachnoid villi and granulations may be an auxiliary system for CSF egress, occurring only in conditions of high intracranial pressure. However, whether the olfactory route is the primary site for CSF absorption in humans remains to be proven, as the specific mechanism for CSF flow in cranial nerves reaching the eyes and nose is still awaiting characterization (Johanson et al. 2008).

## 1.6 THE HYDROCEPHALIC TEXAS RATS ANIMAL MODEL



**Figure 1.11. The image shows a Sprague Dawley pup (animal above, control normal rat) in contrast to a hydrocephalic pup (animal below, Texas rat). The red arrow points to the dome cranium on the hydrocephalic head, which is absent on control head (blue arrow).**

Hydrocephalic Texas rats where first discovered in 1981 in Texas, after observations of sudden death from an unknown cause within a colony. Brother-sister matings from this colony were carried out to establish a breeding colony that gave rise to 23% of newborn pups with hydrocephalus. Hydrocephalic pups had an apparent normal growth until the third week of age but stopped growing after that (Kohn et al. 1981)

Genetic analysis of this colony suggested that Texas rats are the result of an autosomal monogenetic spontaneous mutation (Cai et al. 2000). Jones and colleagues genome-wide linkage studies in 2001 using backcross progeny revealed that there are 4 loci involved in

49

the development of hydrocephalus in Texas rats, although the specific genetic defect still requires clarification (Jones et al. 2001).

Congenital hydrocephalus onset in Texas rats happens between the 17 and 18 gestational days, and it is apparently due to stenosis of the aqueduct of Sylvius and morphological changes of the subcomissural organ (Jones et al. 1988). Discrepancies in these findings were stated by Oi and colleagues in 1998, as they did find negligible changes in the development of periaqueductal structures at gestational day 17 using LEW/Jms rats; (an animal model for inherited congenital hydrocephalus). Oi and colleagues compared Texas rats with LEW/Jms rats' morphological changes for aqueductal occlusion, and reported differences between animal models. Thus, whereas in Texas rats the aqueduct collapses at day 17, in LEW/Jms there is a reduction in number of ependymal cells at the level of the narrowest site of the aqueduct with an intact aqueduct. Hence, it was concluded that the mechanisms for stenosis of the aqueduct of Sylvius in these two types of congenital hydrocephalus models is completely different. Unfortunately, at present, the aqueduct changes during development in humans cannot be properly characterized. Nevertheless, brain development in rodents during the neonatal period is similar to that in humans in the late fetal period, and this means that the Texas rat model is suitable for studying human congenital hydrocephalus. Proof of this is that human fetal hydrocephalus at stage 23 (56 days) shares comparable cerebral cortex abnormalities with Texas rats between embryonic days 17 and 18 (O'Rahilly and Müller 1987, Raimondi et al. 1976 and Kohn et al. 1981).

Texas rats are classified as affected and unaffected depending on the type of changes occurring in brain parenchyma and the presence or absence of brain dilation at gestational day 18. Thus, unaffected rats' brain parenchyma grows similarly to that of normal rats, whilst affected rats present enlarged ventricles and no development of the brain

parenchyma at all (Mashayekhi et al. 2002). Ventriculomegaly in affected rats continues from embryonic day 18 towards the postnatal period (Jones et al. 1998). In addition, cerebral cortex morphological analyses of affected Texas rats demonstrate that the germinal neuroepithelia of these rats is narrower than in normal rats during fetal stages (Jones et al. 1998). Interestingly, reduced cortical thickness is not caused by raised intracranial pressure, as was thought in the past, as this event happens earlier in development, at day 10, instead of day 19 when decreased in brain parenchymal thickness is clearly noticeable (Mashayekhi et al. 2002, Owen-lynch et al. 2003).

Therefore, studies with the Texas rats animal model provided evidence that congenital hydrocephalus is not only related to brain damage caused by elevated intracranial pressure due to CSF built-up in the brain ventricles. In this context, Owen-Lynch and colleagues' studies in 2003 demonstrated that decreased cortical mantle in the affected Texas rats is due to an inefficient radial glia, blocked in the S phase of their cell cycle and unable to proliferate and exert their functions as scaffolding for subsequent generation of enough neurons to populate the upper layers of the cerebral cortex. Histological analysis showed that radial glia cells have elevated levels of DNA, and when these cells were exposed to hydrocephalic CSF "in vitro" proliferation was impeded. This contrasted with normal, CSF which was beneficial for cell growth, and demonstrated the importance of CSF for brain development. Since "in vivo" cerebral cortex cells were arrested in the S phase of the cell cycle but remain able to proliferate "in vitro", a lack of nucleotide availability was suggested. These findings also explain the inability of radial glia to complete the S phase of the cell cycle and progress into mitosis. Since folate metabolism is vital for the production of nucleotides, it was proposed that the folate metabolic cycle may be impaired in hydrocephalic Texas rats. Nabiuni and colleagues' experimental results in 2004, as well as Cain and colleagues' findings in 2009, confirmed this postulate after LCMS and

Western blotting analysis of CSF showed a lack of the folate enzyme FDH in hydrocephalic CSF. Further "in vitro" experiments using cerebral cortex progenitor cells treated with hydrocephalic CSF, showed an increase in cell proliferation when folates and the nucleoside thymidine were added to the media. As a result of these findings, it was concluded that a defective folate metabolic cycle may lead to congenital hydrocephalus caused ultimately by changes in CSF composition and folate protein profile.

## 1.7 TREATMENT FOR CONGENITAL HYDROCEPHALUS

At present, ventriculoperitoneal shunt or ventriculostomy is the conventional method for treating hydrocephalus. This technique involves making surgical insertions of bypass tubes or catheters in the brain and peritoneal cavity to channel brain CSF outflow into the peritoneal cavity, thus improving drainage of obstructed CSF (figure 1.12). Unfortunately, failure rates are high in the shunting techniques and the complication rate in terms of infections and/or shunt failure for the first year of a newborns' life is about 17%. By 2 years of age, this rises to almost 50% (Kazan et al. 2005). In spite of shunting, neurological deficits still remain in hydrocephalic patients, due to the increased intracranial pressure exerted by CSF before shunting (Oreskovic et al., 2010). These facts highlight the necessity of an alternative non-invasive treatment in the future to replace the current methods.
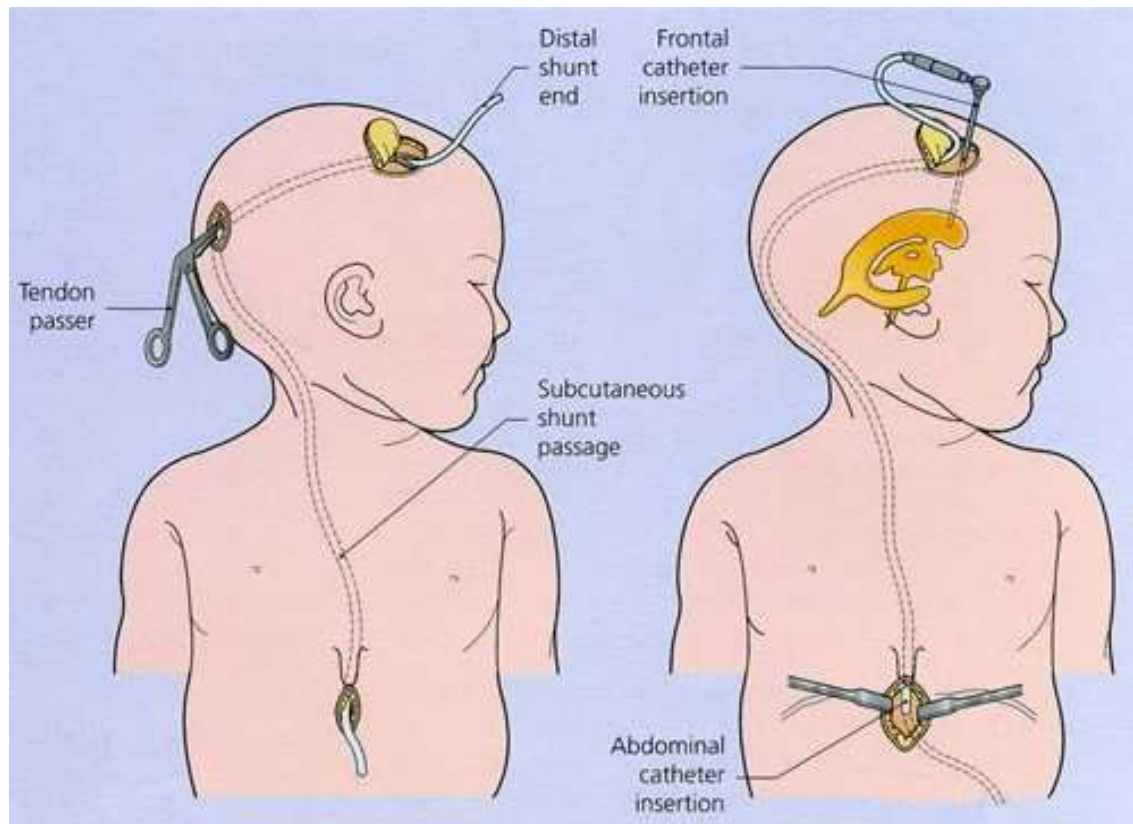


**Figure 1.12. Schematic representation of the ventriculoperitoneal shunt technique. Surgical incisions are carried out at the frontal and parietal bone as well as at the abdominal level, to incorporate a catheter that connects brain ventricles with abdominal cavity for release of obstructed ventricular CSF into the stomach. Adapted from www.seattlechildren.org**

## 1.8 FOLATES/VITAMIN B9.

Folates or vitamin B9 play a vital role in the development of the CNS as they are key molecules for the adequate functioning of the folate metabolic cycle, which is suggested to be deficient in congenital hydrocephalus.

### 1.8.1 Folates and the folate metabolic cycle.



**Figure 1.13. The folate metabolic cycle. Folic acid supplementation and food intake provides the body with dihydrofolate and 5-methyltetrahydrofolate (5-methylTHF) which are converted into Tetrahydrofolate (THF) by Dihydrofolate reductase (DHFR) and methionine Synthase (MS)/methionine synthase reductase (MSR) respectively, with concomitant production of methionine in the presence of vitamin B12 (B12). Methionine synthesis occurs in parallel to DNA methylation by means of methyl groups provided by 5-methylTHF. Methionine is also used to produce S-Adenosyl methionine (SAM), which is a methyl donor in numerous metabolic reactions. THF can be transformed into 5,10-methylene-THF, 5-fmm-THF and 10-formyl-THF. THF, on the other hand, is produced from DHFR. Folinic acid or 5-formyl-THF, along with other monoglutamated folates (MnTHFs) are converted into 5, 10-methenyl-THF, which is transformed into 10-formyl-THF the substrate for FDH. FDH transformed 10-formyl-THF into THF, closing the metabolic cycle (Modified from Molloy and Scott 2001).**

Folates are THF polyglutamate water-soluble cofactors that belong to the B9 vitamin family and are crucial for brain development. They are involved in a huge range of metabolic reactions, where their role is to accept and donate carbon atoms. All folate metabolic reactions are interrelated and constitute the folate-mediated, one-carbon metabolism cycle (Figure. 1.13). This cycle is active in the cytoplasm and in the mitochondria of mammals (Molloy and Scott 2001), and active folates carry one carbon atoms at three oxidation states, ranging from formate to methanol (Wagner 1995). Cytoplasmic folate-mediated one- carbon metabolism is required for the de novo synthesis of purines (provides the carbon-2 and the carbon-8 of the purine ring) and thymidylate (methylation of dUMP to dMTP), as well as for remethylation of homocysteine to methionine. Methionine can be converted to S-adenosylmethionine, which acts as a methyl donor for various methylation reactions (methylation of DNA, RNA and proteins). Further, the main source of folate-activated one-carbon units is serine (Wagner 1995, Shane 1995, Davis et al. 2004 Anguera et al. 2006 and Solansky et al. 2010). Cytoplasmic serine hydroxymethyltransferase catalyzes the THF-dependent aldol cleavage of serine to methylene-THF and glycine. Alternatively, Serine can be converted to glycine and formate in the mitochondria as folate-mediated one-carbon metabolism also takes place there (Suh et al. 2001, Anguera 2006). The formate produced in the mitochondria is a major source of one-carbon units for cytoplasmic one-carbon metabolism through its conversion to 10-formyl-THF by the trifunctional enzyme C1-THF synthase, also called methylentetrahydrogenase-1. This encodes 10-formyl-tetrtahydrofolate synthetase, methylene-THF dehydrogenase, and methenyl-THF cyclohydrolyase activities.

**1.8.2 Folate binding enzymes and the folate metabolic cycle.**

There are two important folate binding enzymes associated with impairment of folate-mediated one-carbon metabolism: Methylen-THF reductase and FDH. Methylen-THF reductase disease is the most common inborn error of folate metabolism and consists of a deficiency in the mitochondrial version of this enzyme leading to severe psychomotor retardation, mycrocephalus and seizure (Garcia-Cazorla et al. 2008 and Megumi et al 2011).

A second important folate binding protein suspected to be involved in deficient folate metabolic cycle is the enzyme 10-formyl-THF dehydrogenase (FDH), also called Aldehyde Dehydrogenase L1 (ALDH1L1). FDH is a soluble protein (Cook and Wagner, 1991) mainly expressed in liver and brain (Girgis et al. 1998 and Hong et al. 1999). Its metabolic role in folate-mediated one-carbon metabolism has not been totally clarified yet (Anguera et al. 2006, Krupenco 2009, and Krupenco, 2010) but cellular in vitro models suggest that FDH may regulate the folate-mediated one-carbon metabolic network in specific cell-types (Min et al. 1988, Anthony and Heintz 2007, Strong and Schirch 1989 and Anguera 2006). Four physiological functions have been attributed to FDH. The first one is its function in removing excess of one-carbon units from folate metabolism by depleting 10-formyl-THF pools (Krebs et al. 1976, Champion et al 1994). Its second function is to facilitate the production of folate-activated one-carbons by channelling THF to enzymes that are located at the primary entry points in the one-carbon metabolic cycle. These enzymes are the cytoplasmic serine hydroxymethyltransferase and the trifunctional enzyme methylene-THF dehydrogenase 1, both of which have an important role in suppressing FDH activity in situations of high THF concentration (Fu et al 1999 and Kim et al 1996). According to Anguera (2006) these functions may be working alternatively, depending on the levels of FDH expression. Anguera also proposed that high levels of

FDH expression greatly reduce the supply of folate activated one-carbon units, meaning that THF becomes the primary cellular folate derivative. In other words, high FDH expression levels mean an increase in the amount of binding site availability for 10-formyl-THF, and this result in depletion of 10-formyl-THF pools through the FDH-catalyzed conversion of 10-formyl-THF to THF. This may cause defective cell growth by depletion of the supply of the one-carbon units in the form of 10-formyltetahydrofolate. At the same time, low levels of FDH expression increase the 10-formyl-THF/folate ratio. This is consistent with the suggested role of FDH in facilitating the generation of folate activated one-carbon units. Since FDH has a higher affinity for THF than 10-formyl-THF in situations of low FDH expression, its activity depleting 10-formyl-THF pools is expected to be lower comparing to its folate channelling activity through cytoplasmic serine hydroxymethyltransferase and the trifunctional enzyme methylen-THF dehydrogenase 1 (Fu et al 1999 and Kim et al 1996, Anguera et al. 2006).

A third function attributed to FDH is its capability to sequester cellular folate and store it in the form of THF in vivo in normal conditions (Fu et al. 1999). The sequestering mechanism is due to the intracellular FDH levels exceeding the THF concentrations. In this scenario, all cytoplasmic THF is expected to be bound to FDH. This matches experimental results from animal models of mice lacking FDH expression, where in spite of a folate sufficient diet; the total folate concentration is reduced (Champion et al. 1994).

Finally, as a forth function, FDH has been suggested to inhibit purine biosynthesis by also depleting 10-formyl-THF pools (Krupenko and Oleinik 2002).

**1.8.3 Known folate transporters and FDH as a potential folate transporter.**

Folate transporter mutations and loss of folate transport in the brain are both linked to cerebral folate deficiency and brain neurodegeneration in childhood (Steinfeld et al. 2009 and Grapp et al. 2014). Furthermore, the presence of folate receptor autoantibodies in plasma is associated with low folate in CSF, and with neurodegenerative disease (Ramaekers et al. 2005). The known folate transporters and the potential role of the folate bind to enzyme FDH are described below.

1.8.3.1 <u>Known folate transporters</u>

The main known folate transporters in the brain are PCFT, RFC, and FRα. They have been characterized in the choroid plexuses; however, their potential expression in other areas of brain is also to be considered (Ramaekers et al. 2004 and Requena Jimenez et al. 2015).

It is accepted that folate transporters bind to soluble folates in blood for later secretion into CSF, with folate concentration in CSF doubling the systemic circulation levels due to the active transport occurring in the choroid plexuses. Folate transporters are only active at specific pHs, and rely on basal membrane transport (Na+/H+ exchange), which is coupled to active transport by ATPases to achieve the suitable intracellular acidity that allows its functionality (Segal 2001, Zhao 2011).

As illustrated in figure 1.14 below, FRα is anchored to the basolateral membrane of the choroid plexus by a glycosylphosphoinositol (GPI) domain, and transports folate via receptor-mediated endocytosis. At the same time, PCFT is also expressed along the basolateral membrane and carries folates from blood into choroid plexus cells in parallel with FRα. It has been also reported that PCFT transports 5mTHF in acidified endosomal compartments into the cytoplasm for later bind to FRα (Solanky et al. 2010).

Whereas FRα transports folates from blood to CSF (Selhub 1984 and Weitman 1992), RFC (SLC19A1) operates bidirectionally and is highly expressed at the apical brush-border of the choroid plexus membrane (Wang et al 2001). Accordingly, it is proposed that folate transporters work in tandem and they are all required for folate transport from blood to CSF (Zhao et al. 2011). A hypothetical model for folate transport including the three transporters is detailed below.
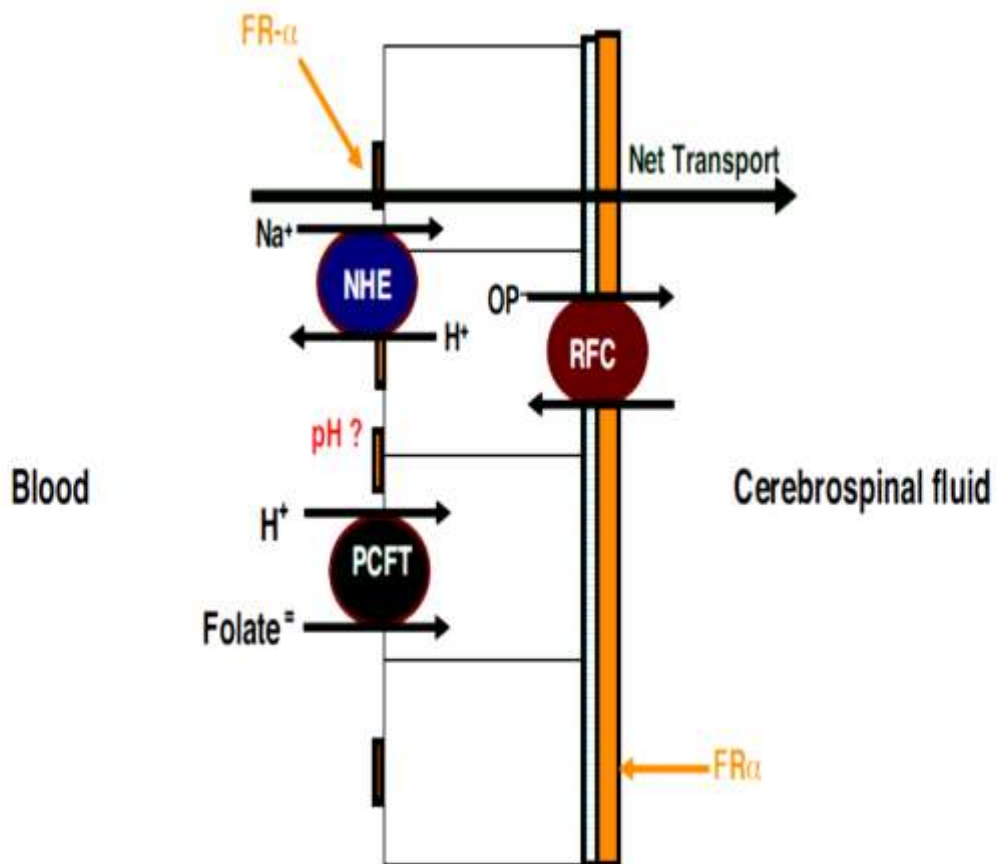


**Figure 1.14. Folate transport across the choroid plexuses in adults. Both the proton-coupled folate transporter (PCFT) and folate receptor (FRα) are key transporters of folates from blood to cerebrospinal fluid across the choroid plexus cells. FRα is mostly expressed at the apical brush-border membrane along with the reduced folate carrier (RFC) whereas PCFT expression and net proton exchange (NHE) are mainly present in the basal membrane in contact with blood (Modified from Zhao R et al. 2011).**

It is suggested that FRα depends on PCFT for folate transport as the former is susceptible to the acidity levels present within vesicles where PCFT transports 5mTHF. Moreover, FRα transport and its high affinity for 5mTHF may depend on folate extracellular concentrations. In this respect, folate transport through FRα seems to happen only at low folate extracellular concentrations, whilst folate transport at elevated folate extracellular levels is carried out by RFC (Zhao et al. 2011). Folate transport has been further described, and it is now believed that FRα is the main folate receptor responsible for folate (5mTHF) transport in the brain. Folate receptor-mediated endocytosis seems to allow transport of FRα-5mTHF complexes through the basolateral membrane of the choroid plexus, and the production of internal vesicles (endosomes) permits shuttling of FRα-5mTHF complexes across the choroid plexus to finally deliver them into CSF within exosomes. Exosomes reach the ependyma bathed with CSF, and provide folate to nearby astrocytes and neurons. See figure 1.15 below.
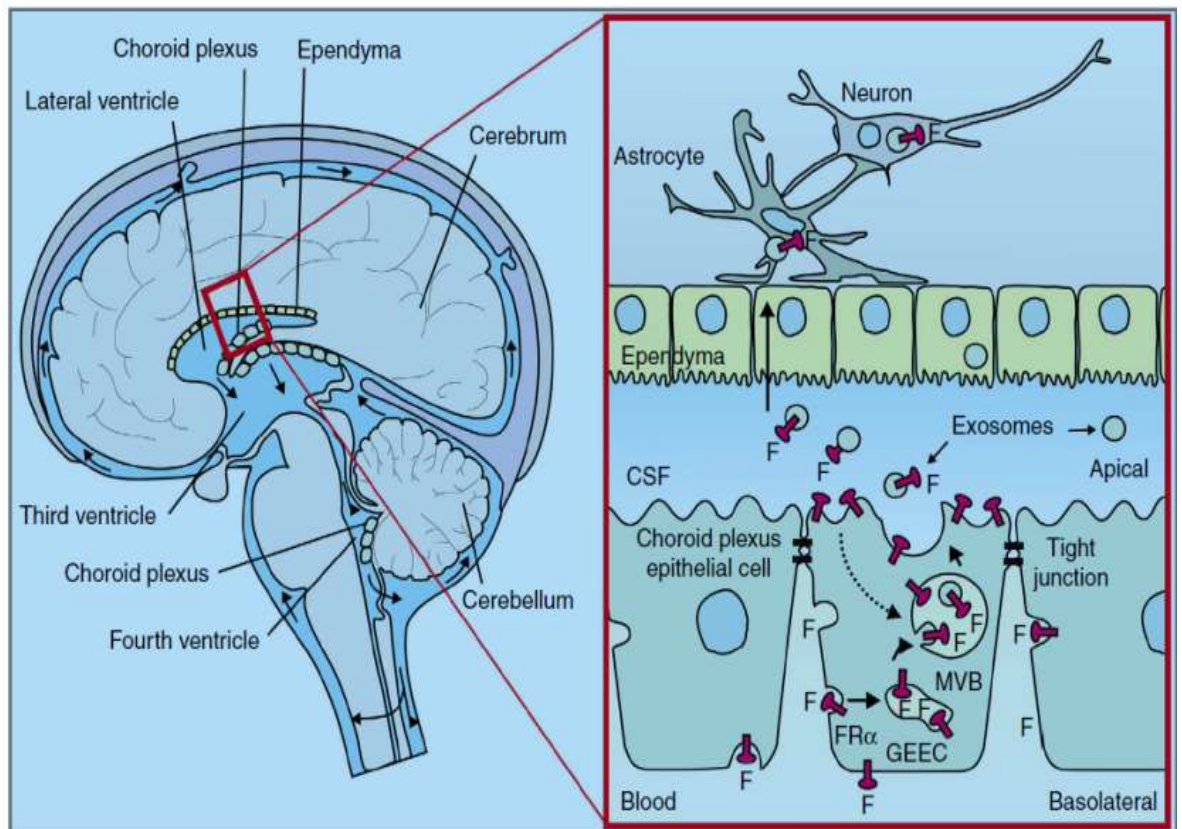
**Figure 1.15. Hypothetical model of human cerebral folate (5mTHF) transport by FRα.** The black arrows show CSF flow and the rectangle indicates the magnified area including the choroid plexus and the ependyma lining the lateral ventricle. The basolateral membrane is in contact with blood circulation, where folates are transported. Folates bind to FRα receptors anchored to this membrane by means of glycophosphatidylinositol (GPI). Afterwards, FRα-folate complexes are internalized through endocytosis, forming GPI-anchored protein-enriched early endosomal compartments (GEECs), and this is followed by translocation of FRα-folate complexes from GEECS to multivesicular bodies (MVBs) or endosomes. Some folate is also transferred to PCFT located within acidic vesicles, to be later transported into the cytoplasm. FRα-folate complexes leave the choroid plexus within exosomes to reach the ependyma and from here to astrocytes in immediate contact, to be later further transported into nearby neurons (adapted from Grapp et al. 2014).

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1.8.3.2 <u>FDH as a folate binding protein</u>

Recent findings in our laboratory showed how the folate enzyme FDH may function as a folate transporter (Krupenco, 2009). FDH consists of three domains, the hydrolase domain, the intermediate or linker domain and the aldehyde dehydrogenase domain (See Figure 1.16). The intermediate domain, or linker, allows the enzyme to be enzymatically active by acting as a bridge between the other two domains (Krupenko 2009). Analysis of the linker sequence using the data base of proteins domains (www.expasy.org/prosite/) suggests that this domain is a member of a group of carrier proteins with a serine bound to a 4-phosphopatheine prosthetic (4-PP) swinging arm. This swinging arm allows the transfer of chemical groups between different domains displayed in multicomplex enzymes such as acyl carrier proteins. Therefore, it is suggested that FDH is a formyl carrier protein able to transport formyl groups from the N domain to the C domain through the linker or swinging arm similarly to acyl carrier proteins. Further, FDH may control in this way the production of THF and 10-formyl-THF pools by formyl transfer between domains (Anguera et al. 2006, Donato et al. 2007). Importantly, it is also thought that the addition of the serine- 4-PP intermediate domain to FDH is a posttranslational modification occurring once the protein is folded and therefore FDH N domain may be found as a single domain within the cells (Donato et al. 2007) before later enzyme modifications.
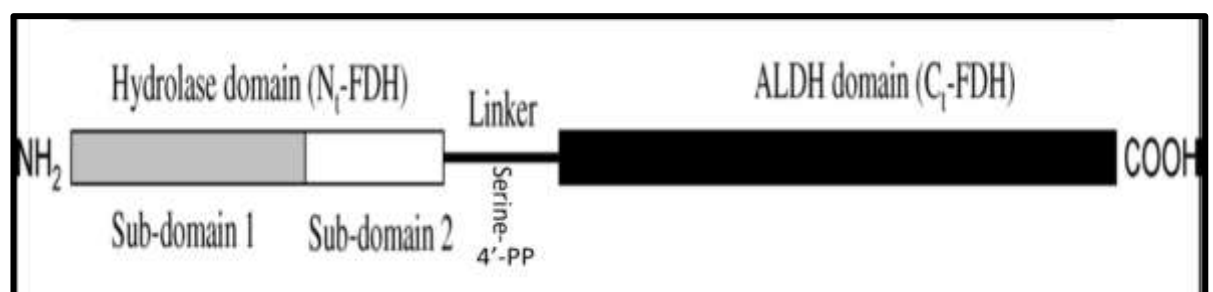


**Figure 1.16. FDH domains. The linker domain is serine dependent and linked to a 4'-phosphopantetheine prosthetic group (4'-PP). The linker or intermediate domain joins the hydrolase domain (N domain) and the aldehyde dehydrogenase domain (C domain), making FDH functionally active.**

Thus, we hypothesize that enzymatically active FDH  may co-exist with enzymatically inactive FDH acting as a mere folate binding protein handling folate to FRα to further transport it to different areas of the fetal brain.

In our laboratory, Sprague Dawley rat brain immunolocalization and Western blotting techniques detected high levels of FDH in cerebral cortex and embryonic CSF before the neural tube closes (day 17 of rat embryonic development). Interestingly, FDH was not detected at the choroid plexuses, and the FDH levels fell dramatically at embryonic day 18. In contrast, in hydrocephalic rats, FDH at embryonic day 18 was not released into CSF, but was immunolocalized at the same site as in normal rats; the cerebral cortex. In CSF, FDH was identified in vesicles (data not published yet) in normal and abnormal rats, and consistent with these results, other research group revealed the presence of CSF vesicles or nanospheres with an unusual membrane. These nanospheres were found in health and disease, and it was suggested they are responsible for humoral signaling mechanisms in the brain (Harrington et al. 2009 and Bachy et al. 2008).

Following our results, we suggest FDH may present an alternate folate transport mechanism to the usual folate transport by folate receptors, and reduced folate carriers. FDH-folate transport may be specific for certain areas of the brain like the cerebral cortex whereas other regions such as the choroid plexuses use preferentially PCFT, FRα and RFC.

1.8.3.3 <u>Folates and folate deficiency</u>

Low folate concentration in CSF is associated with different types of folate deficiency. A first type of folate deficiency is due to hereditary folate malabsorption, which is a condition clinically manifested after birth. Interestingly, this condition involves severe folate transport impairment at the choroid plexuses, and even when the blood folate levels are normal, CSF folate remains very low (Geller J et al. 2002). Folate deficiency can also be the result of mutations of the Folate Receptor 1 gen (FOLR1); FRα, leading to a type of folate deficiency that manifests several years after birth (Steinfeld et al. 2009).

In addition, folate deficiency, specifically 5-methylTHF deficiency, is associated with the presence of autoantibodies in blood against high affinity folate receptors located at the blood brain and blood-cerebrospinal fluid barrier. This type of folate deficiency has important negative repercussions for brain development, as 5-methylTHF is the predominant folate in blood, CSF and food (Ramaekers et al. 2004, and Ramaekers et al. 2005). Decreased 5mTHF levels are also linked to cerebellar ataxia, psychomotor retardation, dyskinesia, severe developmental delay, CNS calcifications, epilepsy and autism spectrum manifestations (Moretti et al. 2008). In our laboratory, recent studies on animal models of hydrocephalic rats revealed a possible relationship between folate imbalance and deficiency of specific folates in CSF (Cain et al. 2009). This finding implies that congenital hydrocephalus is also a type of folate deficiency. Folate deficiency alters dTMP, methionine, and S-adenosylmethionine synthesis, resulting in elevated rates of uracil misincorporation into DNA and DNA hypomethylation. Thus, maintenance of cellular folate concentration is vital for DNA synthesis, stability and expression. At the same time, intracellular folate concentrations depend on folate availability, uptake, polyglutamation, and turnover and export (Suh et al. 2001).

Previous findings in our laboratory from "in vitro" cerebral cortex primary cell cultures also showed that cell proliferation decreases when exposed to embryonic day 20 hydrocephalic CSF. This meant that CSF lacking FDH induced cell cycle arrest. Proliferation assays were used to measure DNA biosynthesis, and it was observed that cells were blocked at the S phase, which explained the inability of progenitor cells/radial glia cells to proliferate and differentiate. Also, stained sections from cerebral cortex showed a poorly developed cerebral cortex, and it was hypothesized that depletion of DNA due to a lack of FDH in CSF may explain the reduced thickness of the cerebral cortex and germinal epithelia observed after embryonic day 18, which coincides with CSF obstruction in the Sylvius aqueduct in the hydrocephalic rat (Mashayekhi et al. (2002). The possibility that components in hydrocephalic CSF may have inhibitory factors for normal cell growth and FDH release was considered.

In conclusion, our data lead to the conclusion that alterations in the expression or function of FDH cause an imbalance in folate metabolism, and abnormal cortical development of the brain (Cains, S. et al. (2009); Moretti P. et al. (2005); Hansen FJ. and Blau N. (2005)).

In relation to folate metabolites, our experiments also showed a decrease in 5-methylTHF in serum samples from adult H-Tx rats when compared with controls (Cains, S. et al. (2009)). However, 5-methylTHF concentration was similar in normal and abnormal foetal CSF, and this result did not lead to any conclusion.

1.8.3.4 <u>Folate treatment</u>

The synthetically produced folic acid (pteroylglutamic acid) is used to fortify food in the USA (Geraldine et al. 2007). Food fortification has proven to be successful in increasing folic acid intakes pre-conceptually, preventing premature delivery, congenital malformations and neural tube defects such as spina bifida (Refsum 2001, Antony 2007, Scholl et al. 1996). Also, common dementia in the elderly is associated with folate and vitamin B12 deficiency. Consumption of fortified food, however, has negative effects for the elderly, as fortified food increases the risk of 'masking' megaloblastic anaemia caused by vitamin B12 deficiency. This is explained by the fact that metabolic functions of folate and vitamin B12 intertwine in the methylation cycle, and they affect each other, and the processes they modulate together (Lindenbaum et al. 1994, Joosten et al. 1997 and Geraldine et al 2007). Folic acid fortification masking vitamin B12 deficiency is also important in vegetarian populations which are deficient in vitamin B12 (Yajnik et al. 2006). Other negative effects of folic acid have been seen in epileptic patients, who experience frequent seizures (Reynolds 1968 and Dennis and Taylor 1969). Finally, folic acid has also been described as a risk factor for strokes (Motulsky, 1996). Interestingly, these investigations are in agreement with our results about the negative effects of folic acid supplementation. Thus, folic acid did not prevented hydrocephalus in pregnant Texas rats and even more, it did increased significantly the incidence of hydrocephalus within the progeny (Cain et al. 2009).

Folinic acid/leucovorin is considered a good alternative to folic acid because it is efficiently transformed into 5, 10-methyleneTHF and 5-methyl THF and has a higher bioavailability than folic acid (Spector, 1979 and Brocker, Bonhomme, Lods 1990). Folinic acid (5-formyl THF) has been used to improve psychomotor development in Down's syndrome children, with success and no side effects despite its intense use during

the treatment (Blehaut 2010). Children with developmental delay and autism presenting low levels of 5-methylTHF have also benefited from treatment with folinic acid in terms of improvement in their motor skills. Nevertheless, their cognitive language and socialization skills remained delayed (Moretti 2005, Steinfeld, R et al. 2009).

In our laboratory, treatment with folinic acid and THF was carried out to study their effect on hydrocephalic rats at embryonic day 20. A mixture of folinic acid and THF at both at 2.25mg/kg successfully reduced the incidence of congenital hydrocephalus by 60%. In contrast and as aforementioned, treatment with folic acid did not decrease the incidence of hydrocephalus. It was proposed that whereas in conditions such as spina bifida, folic acid is needed for prevention before and during the first trimester of pregnancy (Tamura T. et al. 2006), in hydrocephalus, cerebral cortex abnormalities have to be fixed with folates throughout pregnancy, and perhaps a specific folate profile demand may be required by the foetus at different time points during its growth (Cains et al. 2009).

**1.9 SUMMARY.**

Brain Cerebrospinal fluid (CSF) bulk flow is unidirectional and maintained thanks to a balance between CSF secretion from the choroid plexus and CSF absorption by arachnoid villi, where it drains into nearby blood vessels, thereby reaching the general blood circulation. Congenital hydrocephalus starts during the first trimester of pregnancy with impeded CSF flow, and consequent CSF build-up within the brain ventricles. This event is followed by CSF compositional changes, increased intracranial pressure, and, if untreated, brain damage and fetal death. Previous research has revealed a unique folate delivery system which serves the developing cerebral cortex. Abnormal folate provision due to impairment of this system was directly connected to a decrease in a CSF folate enzyme: 10-Formyl-Tetrahydrofolate dehydrogenase (FDH), which is also called aldehydrogenase type 1 (ALDH1L1). This enzyme acts as a folate binding protein and it is believed to be part of the brain mechanisms for folate transport and provision. In light of these findings, low FDH was linked with folate deficiency and the poor cortical development found in congenital hydrocephalus. Furthermore, it was proposed that this disorder is due to a folate deficiency caused by low FDH in hydrocephalic CSF, and that it can be alleviated through maternal folate supplementation. Past studies demonstrated that neither folic acid nor folinic acid supplementation on their own fix congenital hydrocephalus. However, folinic acid in combination with THF did successfully prevent and reduce the incidence of the condition in rodents. In this context, further investigation was carried out in this study to ascertain whether folates in the presence and absence of the folate enzyme FDH are beneficial for fetal brain development. The current study also aims to investigate the FDH - folate delivery system in the fetal brain in order to understand its role in CNS development and its relationship to currently known folate transport mechanisms (FRα). Furthermore, we hypothesize that folates may prevent congenital hydrocephalus through a re-

establishment of CSF drainage and flow circulation at the level of the arachnoid membrane/villi. This assumption implies that the leptomeninge arachnoid may also be dysfunctional in the hydrocephalic brain due to a variation in hydrocephalic CSF composition. Finally, an overall analysis of the constituents uniquely present in normal CSF, hence missing in abnormal CSF, and vice versa, was carried out to establish associations with predicted activated and inactivated biological processes in congenital hydrocephalus, other than impaired folate metabolic cycle.

In general, the present study intends to achieve a better understanding of the roles of FDH and folates, as well as the discovery of other potential key CSF components involved in brain development. It is hoped that this will allow the discovery of key supplements and an adequate treatment for the prevention of a condition that can at present only be treated with invasive surgical procedures entailing life-threatening side-effects for the baby.

## 1.10 EXPERIMENTAL AIMS AND HYPOTHESIS

 Aim and hypothesis 1: **Effect of folates and FDH on cerebral cortex cell proliferation.**

This study aims first to determine if there is a positive effect of folates on cerebral cortex cell division in the presence and absence of FDH, focusing on THF as this folate is proven to be a promising candidate for the treatment of congenital hydrocephalus in Texas rats. THF is an unstable natural folate, and for this reason we intended to stabilize it by linking it to specific fluorescence immunoglobulins using a commercially available kit. Fluorescence dyes may permit tracking of THF inside cells and visual confirmation and quantitative analysis of THF effect on cell growth. In conclusion, we hypothesize that folates, and specifically THF may encourage cerebral cortex cell division. "In vitro" experiments using primary cell cultures exposed to different concentrations of folates and FDH were performed to achieve this aim.

 Aim and hypothesis 2:  **FDH binds to 5mTHF.**

FDH is a folate binding protein that binds to THF and 10-formyltetrahydrofolate. Interestingly, FDH binds to THF, its product of reaction, with 10 times more strength than its substrate 10-formyltetrahydrofolate. However, FDH affinity for 5mTHF has never been studied.  Since, "In vitro" experiments in this research showed evidence that the presence of active ALDH1L in media supplemented with 5mTHF in the range $10^{-4} - 10^{-6}$ M (CSF levels) affects cell growth negatively,  it was hypothesized that FDH may bind to 5mTHF extracellularly, impeding its transport inside the cells. To envisage a possible explanation for this finding, an "in house" ELISA technique was developed and optimized to evaluate whether FDH has any affinity for 5mTHF, our second hypothesis in this study.

<u>Aim and hypothesis 3</u>: **mRNA and FDH protein are expressed in the brain and co-localize with other folate transporters (FRα)**

This project also aims to clarify the folate delivery system to the developing cerebral cortex in the context of the known folate transport by FRα.The experimental approach is to determine the location of FDH protein and mRNA expression, and to measure and compare protein expression quantification in the normal and hydrocephalic brain. Within this aim, we also investigated whether FDH co-localized with FRα, the principal folate transporter in the brain. Sprague Dawley rats are used as control and Texas rats as the animal model for the condition. All animals are studied at embryonic day 18 when CSF starts circulating. The rationale behind this experimental approach is supported by the fact that FDH is a folate bind to protein and therefore may also function as a folate transporter that handles folate with FRα, working in tandem to provide the brain with the most abundant folate in food, 5mTHF. See Figure 1.17 below for hypothesis explanation:
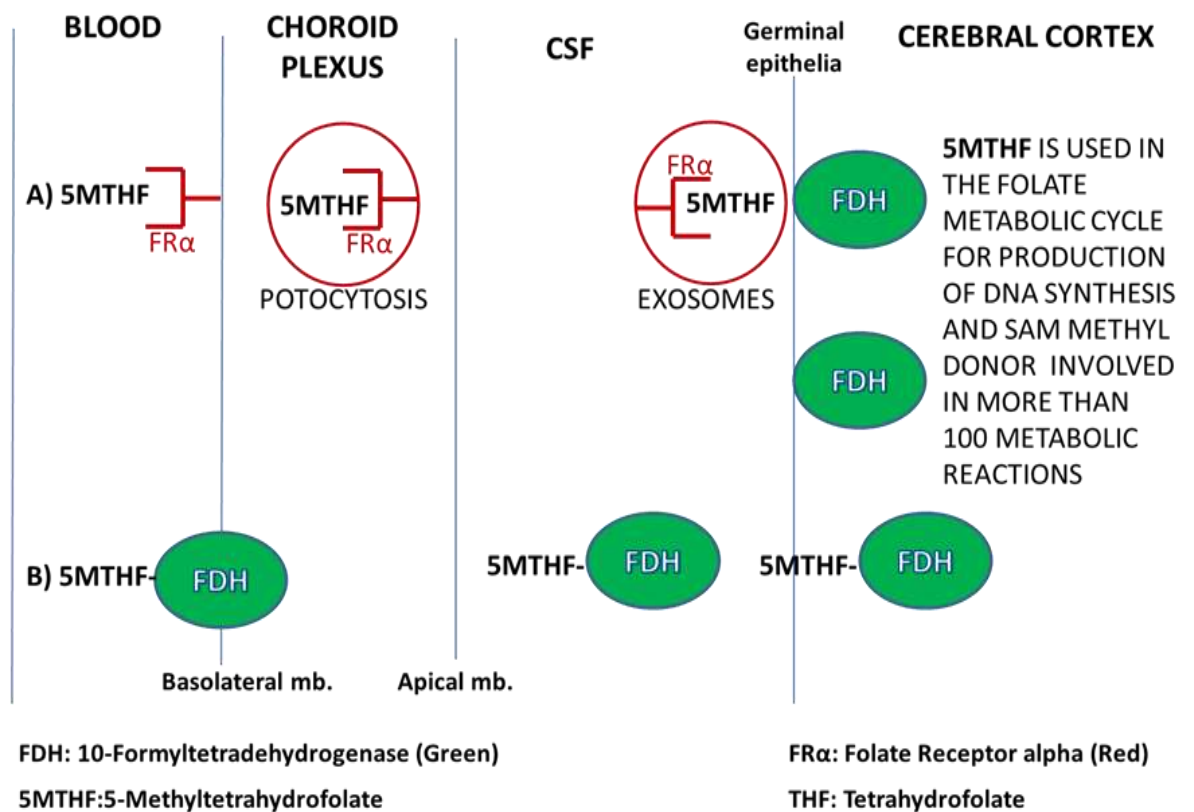
**Figure 1.17. Diagram representing 5mTHF transport by FRα (A) versus our hypothesis related to the role of FDH in folate transport (5mTHF) (B). A) 5mTHF is transported by FRα through the choroidal basolateral membrane by potocytosis (Solanky et al. 2010) 5mTHF-FRα complexes are engulfed by vesicles that leave the choroid plexus to enter the CSF as exosomes (Grapp et al. 2014). Exosomes shuttle 5mTHF-FRα complexes in CSF to later reach the cerebral cortex. 5mTHF is stored or used in the folate metabolic cycle. B) Hypothetical role of FDH in 5mTHF transport from choroid plexus to CSF and within cerebral cortex.**

: **Folates and FDH have an effect on arachnoidal cell growth.**

The role of folates and FDH in arachnoid development is still unknown. Yet, it has been reported that CSF malabsorption due to a dysfunctional arachnoid can lead to congenital hydrocephalus. In this regard, the forth aim of this project is to verify whether folates and folate enzyme FDH have an effect on arachnoid cell growth. A negative effect of folates on cell growth may explain abnormalities in the development of the arachnoid, and inability to divert CSF into the general blood circulation (Figure 1.18)
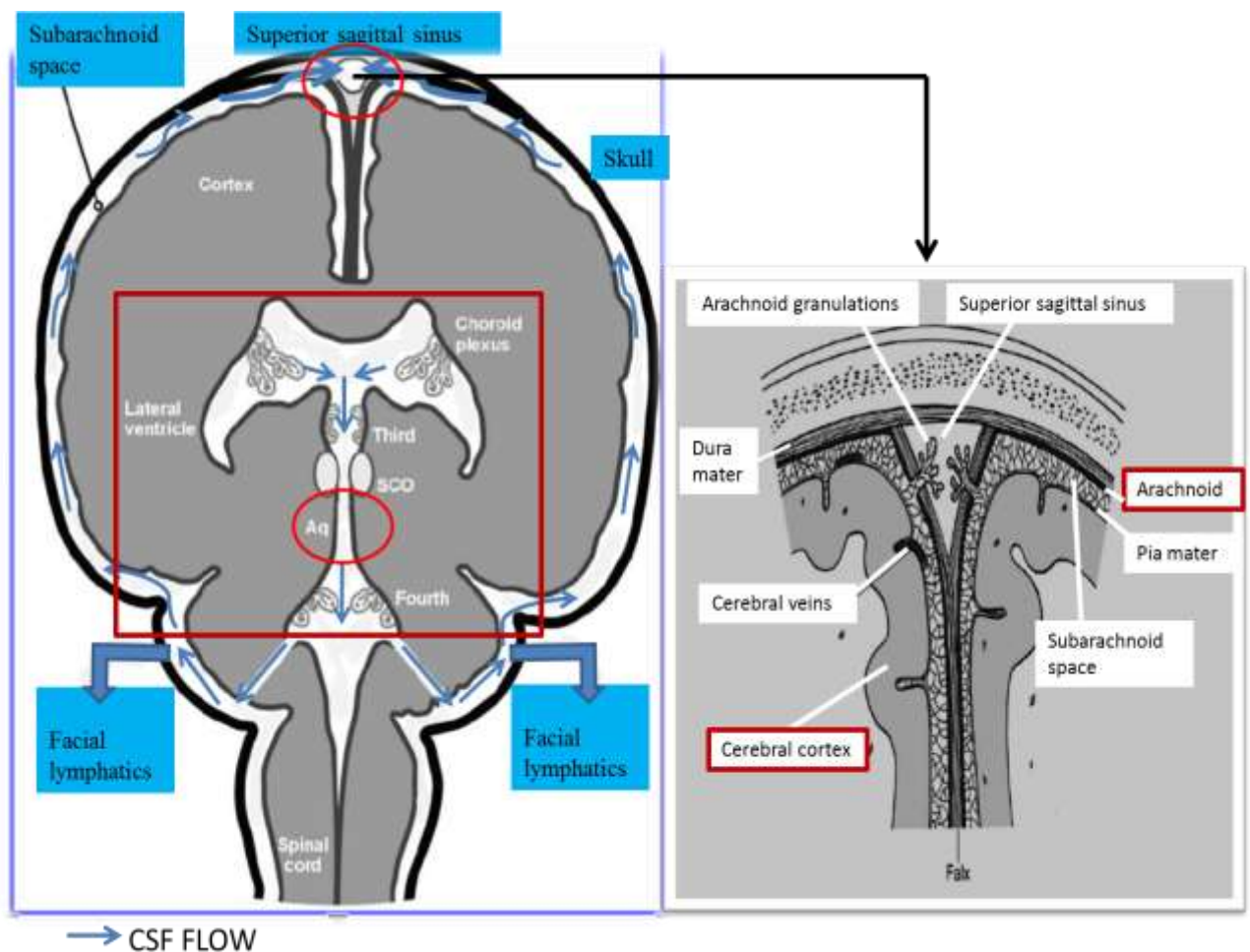


**Figure 1.18. CSF flow and drainage into the superior sagittal sinus. CSF is produced in the choroid plexuses (red rectangle) and reaches the subarachnoid space to leave the brain through the arachnoid, in contact with the superior sagittal sinus. Detailed arachnoid tissue is shown in the second image (black arrow), which illustrates arachnoid tissue projections (arachnoid granulations) towards the superior sagittal sinus where the CSF is drained. Blockage of CSF (top red circle) may be caused by exacerbated growth of arachnoid tissue rendering the arachnoid dysfunctional. The red circle inside the red rectangle indicates a second area of the brain, where CSF flow can be obstructed; the aqueduct of Sylvius. This area is not considered in our studies.**

 Aim and hypothesis 5: **Identification of specific metabolic pathways in normal and hydrocephalic CSF is possible by IPA as well as comparison of metabolic pathway profile between normal and hydrocephalic CSF.**

Past studies related to identification of proteins in normal and abnormal CSF by LCMS revealed a lack of the folate enzyme FDH in hydrocephalic CSF. Thus, we focused our studies on the study of proteins related to the folate metabolic cycle. However, no attention was given to the remaining 485 proteins also missing in hydrocephalic CSF. In the present study, we aim first to group into categories the global proteomic profile obtained from LCMS using EXCEL commands to organize LCMS data-sets into two groups. A first group includes proteins exclusively expressed in abnormal CSF, whereas a second category consists of proteins that are absent in abnormal CSF but present in normal CSF. Excel categories are later analysed by Ingenuity Path Analysis (IPA), which is a bioinformatic tool that allows us to match proteins in CSF with their corresponding genes to predict whether these proteins are related and fall within specific metabolic pathways as well as identifying their potential relationship with specific biofunctions. Our approach here is to consider that genes may be activated or inactivated depending on whether their corresponding proteins are expressed or absent in CSF. The logic behind this approach is that proteins exclusive to abnormal CSF imply activation of certain specific genes in congenital hydrocephalus (genes codifying for these proteins), whilst the missing genes expressed only in normal CSF must be inactivated. IPA analysis, on the other hand, finds relationships between these genes and their involvement in specific metabolic pathways (canonical pathways), whilst linking them to potential biological functions and diseases. In conclusion, we hypothesize that those proteins only present in hydrocephalic CSF imply involvement of the corresponding metabolic pathways where these proteins fall. In other words, activation of these metabolic pathways may be linked exclusively to congenital

hydrocephalus, and this information may prove useful in the future for development of a

clinical tool for identification, diagnosis and prevention of congenital hydrocephalus.

## 1.11 ALTERNATIVE FORMAT

This thesis is presented in the alternative format following the rules and regulations of the University of Manchester. It focuses on the general role of folate in normal and hydrocephalic brain development, and the experimental work was structured in relation to three main aspects of this theme:

First, we aim to understand the role of vitamin B9 (folates) in the cerebral cortex, its location along with the key folate enzyme FDH and folate receptor (FRα) and the potential similarities and interrelationships between enzyme and receptor. Second, we aim to examine the effect of folates in arachnoid cell proliferation "in vitro", to determine whether extracellular folate imbalance results in changes in normal cell arachnoid growth, which may lead to arachnoid dysfunction. Finally, an analysis of CSF's protein composition was undertaken to explore the potential metabolic pathways involved in the development of congenital hydrocephalus with reference to the normal brain. Each aim of this research has a corresponding chapter and each chapter is an intended article for publication as follows:

1) **Role of FDH and vitamin B9 in brain development at early pregnancy: implications for congenital hydrocephalus.**
2) **Vitamin B9 (5mTHF) is crucial for fetal meningeal arachnoid cell growth: implications for congenital hydrocephalus.**
3) **Normal versus hydrocephalic CSF Ingenuity Path Analysis (IPA) of proteins profiles obtained from LCMS.**

The three articles as a whole contribute to characterizing the role of FDH and variants of vitamin B9 during brain development in normal and hydrocephalic conditions, and hence they are all related in this respect. Each article's introduction and conclusion is written with the aim of establishing links between all papers. For instance, the findings in  article 1

support the results of  article 2 and 3. In this way, all three articles are linked together as cohesive piece of work. The relevant material and methods are described for every corresponding chapter.

The vast majority of the work in these articles is the result of my own original thinking, experimental design and assay development. My project ideas, experimental work and experimental outcomes were always discussed with my main supervisor, Dr. Jaleel Miyan, who guided me throughout this project.

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# CHAPTER 2

# FDH AND FOLATES ROLE IN BRAIN DEVELOPMENT AT EARLY PREGNANCY: IMPLICATIONS FOR CONGENITAL HYDROCEPHALUS

## ABSTRACT

Past investigations revealed an association between decreased concentrations in CSF of the soluble folate binding enzyme 10-formyl-THF-dehydrogenase (FDH) and reduced cerebral cortex development in the fetal condition congenital hydrocephalus. We hypothesize that it is FDH deficit in the brain which leads to insufficient folate brain transport, causing abnormalities in cerebral cortex progenitor cell division and prompting the cortical plate to diminish in thickness. Because the cerebral cortex plays a key role in vision, audition, memory, language and perception, developmental cerebral cortex aberrations can only have negative implications for the correct functioning of the brain later in life, leading to cognitive and neurological impairment. Thus, the current study aimed to investigate the role of FDH in folate transport and its contribution to the development of the fetal cerebral cortex.

## 2.1 INTRODUCTION

Congenital hydrocephalus is a medical condition which is described as an accumulation of cerebrospinal fluid (CSF) in the brain ventricles caused by blockage of CSF flow (Miyan et al. 2003, and Bulat and Klarica 2010). CSF is constantly produced in the choroid plexuses (Speake et al. 2001) and leaves the brain mainly via the meninge arachnoid. Arachnoid villi are in contact with the venous sinus where they drain into the general blood circulation system (Bulat and Klarica 2010). CSF flow impediment is thought to be the result of one or a combination of the following problems: brain infection during pregnancy (Kaiser et al. 1985 and Ajzenberg et al. 2002), dysfunctional arachnoid (Boulton et al. 1999 and Grzybowski et al. 2006), genetic factors such as X-linked aqueductal stenosis (Fryns JP, et al. 1991, Jones et al. 1997 and Zhang et al. 2006) and more recently, and the object of this research, folate deficit (Cain et al. 2009). Hydrocephalus leads to ventricular dilation, increased intracranial pressure and brain parenchymal damage (Brocker et al. 1990 and Bergsneider 2001), occasioning CSF accretion inside brain ventricles and fetal death if CSF is not removed (Oreskovic & Klarica 2011). Although the pathology of congenital hydrocephalus is still unknown, a growing body of evidence reveals folate deficiency as a presumptive cause for the malformation of the central nerve system during fetal development, in some cases being hereditary and due to folate malabsorption (Denis et al. 1969, Brocker et al. 1990, Geller et al. 2002, Geraldine et al 2007 and Cain et al. 2009, Hyland et al. 2010).

The vitamin B9 family consists of enzyme cofactors called folates. These are polyglutamated one-carbon molecules vital for the correct functioning of the folate metabolic cycle (Antony 2007). Past experiments in our research group described congenital hydrocephalus as a disorder caused by an impaired folate metabolic cycle. Hence, a treatment based on a mixture of folates (folinic acid and tetrahydrofolate (THF) was used to bypass the defective site of the cycle. The treatment reduced the incidence of

congenital hydrocephalus in the progeny to 60% (Cain et al. 2009) and improved cerebral cortex development. This brain area is narrowed in thickness during the condition due to defective cell cycle control (Mashayekhi et al. 2002 and Owen-Lynch et al. 2003). However, whilst spina bifida and cerebral folate transport deficiency conditions are successfully treated with folic acid and folinic acid respectively, congenital hydrocephalus was not ameliorated by any of these folates, on its own, in our previous studies. Following these results, we propose THF as the folate responsible for the reduced incidence of hydrocephalus with our previous treatment, and consequently the study of THF is an important aspect of the present study.

In relation to FDH, it is known that this multifunctional enzyme converts 10-formyl-THF to THF in a NDPH fashion. This reaction regulates intracelullar 10-formtl-THF/THF pools (Champion et al. 1994), thereby controlling *de novo* purine biosynthesis (Krupenco et al. (2002) and Oleinik et al. 2005), and affecting the methylation potential of the cell (Anguera et al. 2006). These functions are only observed when the N and C domain are brought together in one polypeptide by the intermediate domain, a domain which is proposed to have a similar sequence to carrier proteins with a 4-PP swinging arm. It is also reported that the FDH N domain is solely responsible for the protein binding to folates, and that attachment of the N domain to the C domain through the linker is a posttranslational modification (Krupenco et al. 2002 and Krupenco et al. 2005). These characteristics suggested that single-N domains may be found bound to folates in cytoplasm in early embryonic days, and it was proposed that at this stage FDH may function as a mere folate binding protein (Donato et al. 2007).

Past experimental results in our research group pointed to the folate enzyme FDH, as the key folate enzyme responsible for folate deficit and the folate metabolic cycle impairment

observed in congenital hydrocephalus, which leads to variations in CSF composition in terms of folate profile. The resulting folate CSF imbalance was linked to the total absence of FDH in this body fluid (Nabiuni et al. 2006 and Cain et al 2009). Thus, our previous research focused mainly on CSF analysis, comparing normal and abnormal brains, rather than investigating the problem at tissue level. In this context, this research aims to further study FDH function as a folate regulatory enzyme, as well as its role in folate handling along with folate transporters at histological level.

Thus, we first aimed to investigate what specific folate-types are involved in cerebral cortex development in the presence and absence of FDH, focusing on the folate binding properties of the enzyme. Special attention was given to THF as it was the folate candidate chosen in our previous treatment and a vitamin at the core of the folate metabolic cycle. Since FDH binds to THF, its product of reaction, with tenfold more affinity than 10-formyltetrahydrofolate, its enzymatic substrate (Schirch et al. 1994), it is suggested that FDH acts as a THF reservoir for its later use in the folate metabolic cycle (Kin et al 1996, Fu et al. 1999 and Anguera et al. 2006). Moreover, because of THF's vital role in the production of key DNA components, such as nitrogenic bases as well as the amino acid methionine and S-adenosylmethionine (Krebs et al. 1976), it is expected that any variation in either extracellular or intracellular FDH concentration may be detrimental for folate metabolic cycle activity, resulting in compromised brain development. For this reason, it was important to assess FDH and THF effects on cell proliferation. Additionally, THF is a vitamin which is highly susceptible to oxidization, and exposure to air renders it inactive if not protected through stabilization by means of conjugation to structurally complementary molecules in hypoxic conditions (Krebs et al. 1976, Shane 1995, Ramaekers 2004, Davis et al 2004 and Krupenco 2009). Noble gases like Argon, a gas which reduces oxygen concentration to very low levels in hermetic areas (Krebs et al. 1976), was used for THF

conjugation to a specific immunoglobulin G (IgG). THF-IgG experiments were performed to demonstrate whether or not THF has a direct effect on cell growth. At present, it is not known whether the success of the previous treatment on hydrocephalic rats (THF + Folinic acid) was due to THF alone or its mixture with folinic acid. Since folinic acid did not have a positive effect on growth on its own (Cains et al. 2009), we wanted to determine whether stable THF is enough for potentiating cerebral cortex cell growth, thereby confirming its suitability for a future treatment, a second aim of this research.

In spite of the THF's crucial role in metabolism, the most abundant folate in food and body fluids is 5-methyl-THF (5mTHF), a vitamin which is in high demand during the first trimester of pregnancy for development of the central nervous system (Ramaekers et al. 2004, and Ramaekers et al. 2005, Solansky et al 2010 and Reynolds 2014). Yet, 5mTHF cannot be used directly by brain cells and needs to be converted to dihydrofolate and THF in the folate metabolic cycle (Molloy et al 2001 and Kamen et al. 2004). Also, since 5mTHF is found alongside FDH in CSF, and as ALDHL1 is a folate binding protein, we were also interested in demonstrating whether 5mTHF binds to ALDHL1, our third objective in this research. An FDH characteristic that should be highlighted in this study is the fact that this soluble protein when free in body fluids is able to bind to folates very early in its biosynthesis (Donato et al 2007 and Krupenco et al 2009). Thus, FDH behaves at early stages of brain development mainly as a folate binding protein rather than a hydrolase and/or aldehydrogenase. Consequently, a fourth aim of this project is to assess FDH affinity for 5mTHF in order to discover plausible ALDHL1 functional similarities to soluble folate binding proteins like Folate Receptor Alpha (FRα), (Selhub et al. 1984). FRα is a vitamin transporter of great importance in the provision of 5mTHF to the cerebral cortex (Zhao et al. 2011) during development, and is responsible for 50% of the folate transport in the brain (Anguera et al. 2006 and Steinfeld et al. 2009). In this regard, CSF

devoid of ALDHL1 may explain not only folate imbalance in CSF during hydrocephalus, but also a reduced folate delivery downstream with negative repercussions for brain development as a whole.

Summarizing, we focused first on folate's impact on cerebral cortex proliferation in the presence and absence of FDH. Secondly, we concentrated on the effect of natural versus stabilized THF on cell division to ultimately explain and improve treatment efficiency. Thirdly, we studied for the first time FDH folate binding affinity for 5mTHF, and finally we assessed FDH expression and location in normal and abnormal brains in relation to 5mTHF and FRα.

With these investigations, we intend to contribute to broadening knowledge about folate handling mechanisms by FDH, as well as its interrelationships with folate transporters in normal and hydrocephalic brain, and ultimately to provide a potential treatment based on supplementation to the mother of just stabilized key folate (THF). Stable THF may allow a plausible increase in treatment efficiency when compared to the previously developed treatment (THF and folinic acid), overcoming the problem of working with a vitamin which is known to be highly sensitive to oxidation. Even more importantly, stabilized THF treatment will avoid the need for life-threatening and invasive procedures which are the only form of cure at present. The risk of complications associated with these procedures in infants within the first weeks of life highlights the need for a non-surgical treatment like stabilized THF (Greenberg et al 2001, Kazan et al. 2005 and Warf et al. 2009).

## 2.2 MATERIAL AND METHODS

### 2.2.1 Cell growth with folates in the presence and absence of FDH

Cortical progenitors were prepared as previously described (Owen-Lynch et al. 2003). Cells were plated in poly-D lysine-coated 96-well plates at a density of 1 x $10^5$ cells/ml in standard commercial Neurobasal media contains 4mg/L of folic acid, and therefore, for the purpose of our studies, a tailor-made media without folic acid was purchased and supplemented with 1% B 27, without vitamin A (Invitrogen, Paisley, UK) for the specific growth of embryonic cerebral cortex cells. The cultures were maintained at 37 °C in 5% carbon dioxide for 24 hours to allow us to study the cells' response to media containing 5mTHF, THF and folic acid at concentration range ($10^{-4}$-$10^{-9}$ M) found in human plasma (Pietrzik et al. 2010), with and without inactive ALDHL1 (point mutation in intermediate domain) and active FDH (intact intermediate domain) at constant concentration ($10^{-5}$ M) found in CSF by Western blotting (Cain et al. 2009). Inactive and active FDH was kindly provided by Dr. S Krupenco in the department of biochemistry and molecular biology, Medical University of South Carolina, Charleston, SC. Regarding inactive FDH; it was used to study exclusively its folate binding properties in isolation avoiding its enzymatic function, hence only the N domain was active to bind to folates (Fu et al. 1999). The effect of constant 5mTHF specifically at $10^{-8}$ M along with decreasing ALDHL1 concentration at range (0.3-10 x$10^{-5}$) was also determined as well as the effect of inactive and active FDH at concentration range ($10^{-4}$-$10^{-9}$). Media without folic acid was used as negative control. Cell proliferation was measured using the fluorescent PrestoBlue cell viability reagent kit following the company's protocol (Invitrogen, Paisley, UK). Briefly, PrestoBlue reagent was 1/10 diluted in deionized water and 10µl of solution added to 100µl media per well. This step was followed by 30 minutes incubation at 37°C. Fluorescence signal due to enzymatic activity, and therefore cell division was measured on a Magellan 200

fluorimeter (TECAN, UK) set up at 535 nm and 615 nm for wavelength excitation and emission respectively. All folate solutions were purged with Argon before adding to the neurobasal media to avoid folates' oxidation, which renders folates unstable and inactive.

## 2.2.2 DAPI staining of cell cultures exposed to folates in the presence and absence of FDH.

DAPI staining was performed for imaging and visualization of "in vitro" cell growth carried out to assess the effect on cell proliferation of FDH and folates (paragraph 2.2.1). Blue-fluorescent DAPI nucleic acid stain (Invitrogen, Paisley UK) was done following the company instructions, and cortical progenitors cells were prepared as previously described (Owen-Lynch et al. 2003). Briefly, cells were plated in poly-D lysine-coated 96-well plates containing 5 mm glass round coverslips (VWR International, Ltd.) where cells attached and grew at a density of 1 x $10^5$ cells/ml in Neurobasal media without folic acid supplemented with 1% B 27 without vitamin A (Invitrogen, Paisley, UK). Cultures with and without folates were maintained at 37 °C in 5% carbon dioxide for 24 hours. DAPI stains preferentially dsDNA giving rise to fluorescence enhancement detected by a fluorescence microscope with a violet filter. Coverslips were removed from wells, and mounted on slides. Photos were taken from cells attached to coverslips using a Leica DMLB fluorescence microscope connected to a coolsnap digital camera (Princeton Scientific Instruments, Monmouth Junction, New Jersey, USA).

**2.2.3 Cell growth in response to folate (THF) conjugated to IgG.**

2.2.3.1 THF-IgG effect on cell growth and cell quantification using DAPI fluorescence analysis

A study into the effect of THF on cell proliferation, but on this occasion linked to immunoglobulin G (IgG), was carried out to promote THF stability. This experiment was carried out previously to the fluorescence THF tracking experiments explained in paragraph 2.2.3.2., where THF is this time linked to a fluorescent anti-THF IgG for fluorescent tracking. Thus, in this experiment THF was first linked to IgG as follows: THF was attached to anti-THF immunoglobulin G immediately before adding it to the media as described by Leamon et al. in 1991. Briefly, THF metabolite was dissolved in dimethylsulfoside (DMSO) and incubated with a 5 fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 30 minutes at 23°C in the dark to "activate" the metabolite. 1mg/ml of THF antibody was added to a 10-fold molar excess of the "activated THF" and the labelling reaction was allowed to proceed at 23°C for 16h. Unreacted material was separated from the labelled THF using a Sephadex G-25 column equilibrated in PBS at pH 6.7. The extent of THF conjugation to THF antibody was determined spectrophotometrically by measuring the difference in absorbance between conjugated and non-conjugated THF at 297nm in PBS (pH 6.7). Overall, 2 folates were covalently link per IgG. Absorbance was measured on a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Inc).

THF alone and THF-IgG conjugates were added to the media at $10^{-9}$ M, a suitable concentration for cell growth known from previous experiments. Cultures were DAPI stained as detailed before in paragraph 2.2.2. Fluorescence signal due to binding of DAPI to dsDNA correlated with cell number, and the signal was measured using a fluorimeter set

up according to the fluorescence excitation and emission profile for DAPI-dsDNA complexes: 358 nm excitation and 461 emissions respectively. The relative fluorescence index was plotted versus type of media i.e.: with and without THF after 24 hours growth.

### 2.2.3.2 THF- IgG fluorescent tracking

To visualize and confirm whether THF enter the cells, the following experimental procedure was undertaken: cells were plated in poly-D lysine-coated 96-well plates at $10^2$ cells/ml in neurobasal media. Neurobasal media does not contain folic acid, and it was supplemented with 1% B 27 without vitamin A (Invitrogen, Paisley, UK) to maintain cells undifferentiated. The cultures were kept at 37 °C in 5% carbon dioxide for 24 hours to study the cells response to media containing THF bound to fluorescent anti-tetrahydrofolate immunoglobulin G.  Fluorescent THF antibody was prepared using 1 mg/ml THF antibody (Millipore) linked to cyanine dye. Linking was performed using the lightning link Cy3 conjugation kit following the company's protocol (Novus Biological, UK). The folate was attached to the fluorescent THF immunoglobulin G immediately before adding it to the media, and unreacted material was removed from the labelled THF as previously described in paragraph 2.2.3.1. Assessment of the extent of THF conjugation to fluorescent THF antibody is also described in this paragraph. THF-IgG complexes were added to the media at 25µg/ml. Snapshots were taken using a Leica inverted fluorescence microscope (Deirdre Bioimaging unit, University of Manchester) set up at a maximum absorbance of 670 nm suitable for Cy3.

### 2.2.3.3 Bioavailability study of primary cell cultures in the presence of THF-IgG

To investigate if conjugated THF had any effect on cell proliferation during the fluorescent tracking experiments, cell bioavailability was measured in parallel using the fluorescent

PrestoBlue cell viability reagent kit (Invitrogen, Paisley, UK) as described before. Fluorescence signal correlates with enzymatic activity and it was measured on a Magellan 200 fluorimeter (TECAN, UK) set up at 535 nm and 615 nm for wavelength excitation and emission respectively. Fluorescence measurements versus type of media containing THF alone and IgG-THF complexes at the start of the experiment (t=0) and after 24 hours growth were recorded and plotted in graphs.

## 2.2.4 FDH-5mTHF "in house" sandwich ELISA assay development

2.2.4.1 <u>Production of commercial and non-commercial FDH standard curves to quantify FDH binding to solid phase.</u>

To determine the potential FDH affinity for 5mTHF, an "in house" ELISA assay was developed (Figure 2.1 below). A first step consisted of producing FDH standard curves utilizing an FDH ELISA commercial kit (Cusabio Biotech CO). The same kit was used to create a second ALDHL1 standard curve using a non-commercial FDH protein. Non-commercial ALDHL1 was added in cell culture, and for this reason its affinity for 5mTHF was also assessed to ensure this protein had a similar behaviour to the standard protein provided in the commercial kit. Non-commercial ALDHL1 was kindly provided by Dr. S Krupenco. Commercial and non-commercial FDH were full length proteins, and their corresponding standard curves were created following the company's instructions provided in the kit. Figure 2.1 below shows kit's sandwich enzyme immunoassay diagram used for quantification of rat FDH. FDH top standard was used at $2 \times 10^7$ pg/ml and two fold serial dilutions were carried out to create the standard curves. The kit limit of detection was 10.93pg/ml. Fluorescent 5mTHF antibody was prepared using the Lightning Link Cy3 conjugation kit following the manufacturer's protocol (Novus Biologicals, USA).
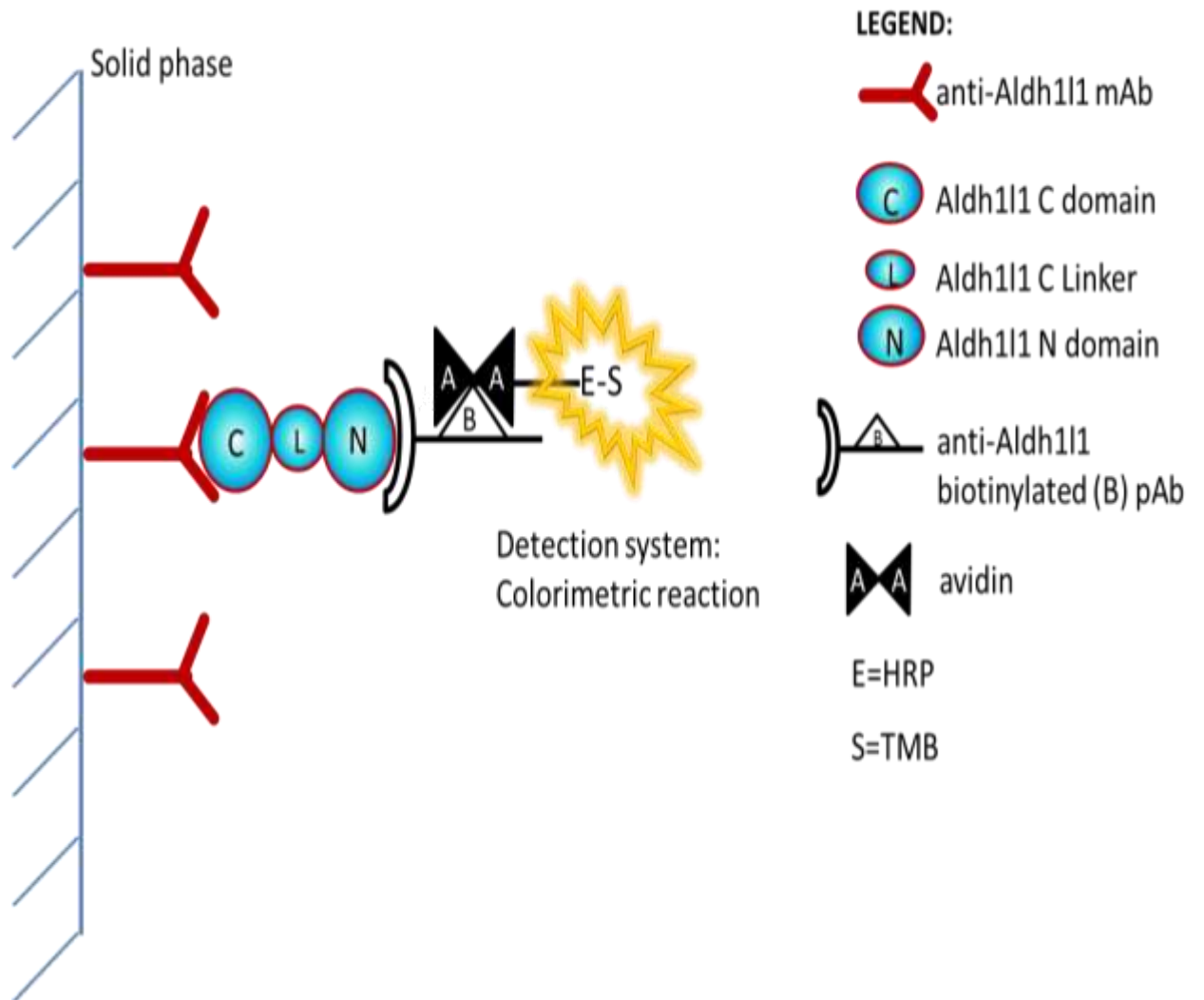
**Figure 2.1. Commercial sandwich ELISA for quantification of rat FDH/ALDH1l1.**

2.2.4.2 <u>Preparation of fluorescent 5mTHF antibody to be used in an "in house" fluorescent ELISA</u>

Fluorescent 5mTHF antibody was prepared using the Lightning Link Cy3 conjugation kit following the manufacturer's protocol (Novus Biologicals, USA).

2.2.4.3 <u>Determination of 5mTHF binding to FDH using an "in house" fluorescent ELISA</u> .
A second ELISA was carried out in parallel to the production of FDH standard curves (paragraph 2.2.4.1.) Thus, the first step in this second experiment aimed to ensure binding of FDH to the solid phase using the conditions suggested in kit, i.e. incubation of solid phase with FDH for 2 hours at 37°C.  FDH attached to the solid phase was then used to ensure detection of potential 5mTHF binding to the enzyme. Therefore, a second step in this experiment was to incubate FDH with 5mTHF overnight, while rocking at 600 rpm. 5mTHF was added at same concentration as FDH, i.e. 2 x $10^7$pg/ml. A two fold serial dilution was performed to obtain a graph representing 5mTHF –FDH linking. 5mTHF and FDH concentration's values chosen for the standard curve were decided according to FDH and 5mTHF data found in the literature for CSF (Cains et al 2009). Also, these values were decided accordingly to experiments performed by Dr. Krupenco using a ternary complex assay based on González-Segura studies in 1993 (data not published yet). After incubation, 5mTHF-FDH complexes were identified using an "in house" fluorescent anti-5mTHF Ab. See Figure 2.2 below for an "in house" 5mTHF-FDH ELISA system.
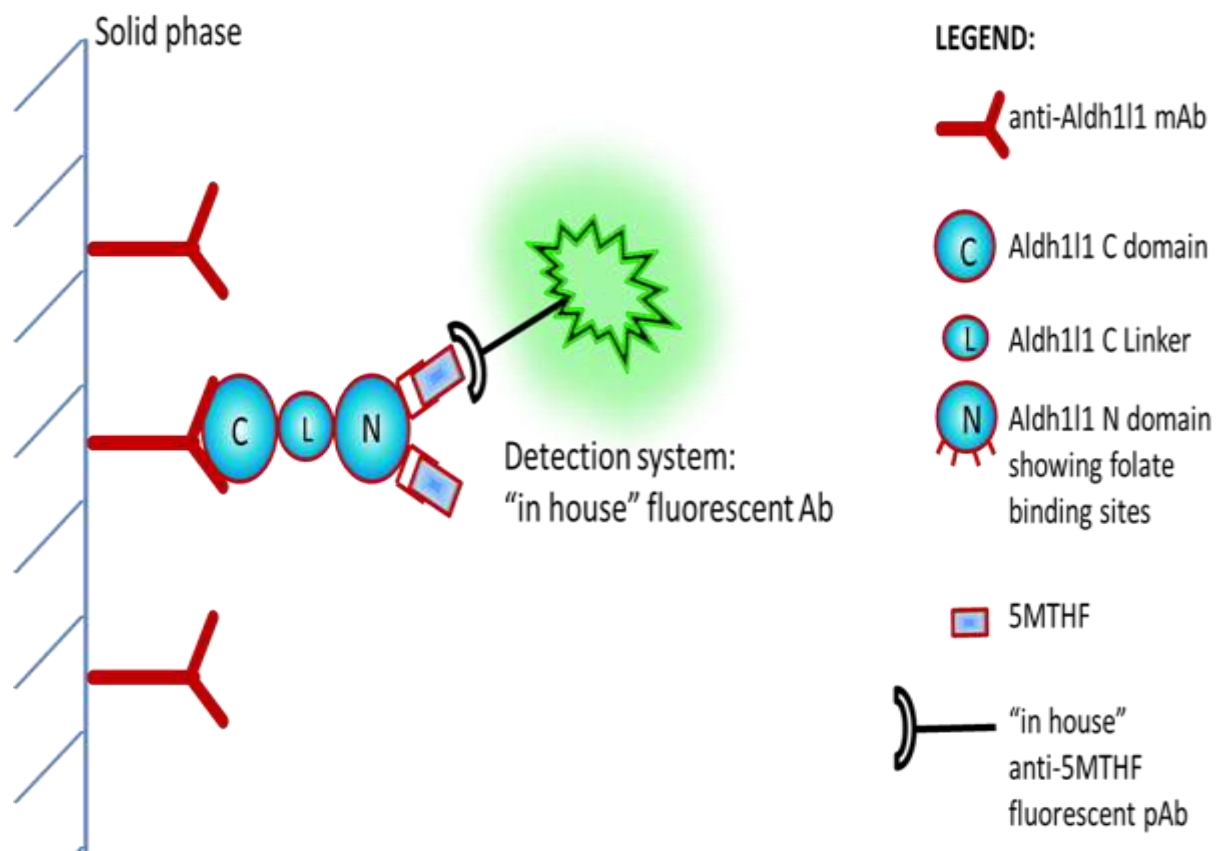
**Figure 2.2. "In house" ELISA assay to identify FDH-5mTHF complexes.**

**2.2.5 FDH, 5mTHF and FRα immunolocalization in normal and hydrocephalic brain.**

2.2.5.1. <u>Fluorescent dual immunostaining of coronal brain sections</u>

Cryosections from gestational day 18 were probed with the following primary antibodies: rabbit anti-human FDH (a gently gift by Dr. S. Krupenco, mouse anti-rat 5mTHF (Millipore) and sheep anti-mouse FRα (R&D Systems) diluted at 1:1000, 1:2500 and 1:20 respectively in chicken serum. Sections were washed by immersion in PBS containing 0.1% Triton-X100 (VWR International, Lutterworth, UK) for 3 X 2 minutes and then antigen retrieved with sodium citrated buffer in microwave until boiling. Slides were allowed to cool and later washed 3 x 2 minutes in PBS. Afterwards, sections were carefully dried and an Immedge pen (Vector laboratories, Peterborough, UK) was used to draw a ring around every section to create a hydrophobic barrier around the edges ensuring this way blocking and Abs solution stayed over the section when added. Sections were blocked to avoid unspecific Ab bind to   for 1 hour in a mixed of goat and donkey serum (Sigma Aldrich Company, Ltd, Dorset, UK) at 5% each serum in PBS-Triton-X100 0.1%. Blocking was carried out at room temperature in a humidity chamber. After blocking, 3 x 5 minutes washes in PBS Tween-20 1% sections were performed followed by incubation primary antibodies overnight. Double immunostaining was achieved by incubating FDH antibody either with 5mTHF or FR. After incubation, 3 x 5 minutes washes in PBS Tween-20 1% were carried out followed by incubation this time with secondary fluorescent Abs. All secondary antibodies were diluted at 1:200 in PBS Tween-20 1%. Green goat anti-rabbit antibody was chosen for FDH (Alexa 488 Sigma Aldrich Company Ltd. Dorset UK), red fluorescent sheep anti-mouse (Alexa fluor 594 Sigma Aldrich Company Ltd. Dorset UK) was chosen to detect 5mTHF and red fluorescent donkey anti-sheep for FRα location (Northern lights R&D Systems, UK). Negative controls were obtained by incubation with PBS instead of primary Abs. Secondary Abs were added to all slides and

incubated for 1 hour. Incubation was followed by 3 x 5 minutes washes in PBS Tween-20 1% before mounting slides in Vectashield with Propidium iodide (Vector laboratories, Peterboroug, UK).

2.2.5.2. <u>Image analysis of sections captured by fluorescence microscopy</u>

Tissue sections were captured on a Leica DMLB fluorescence microscope connected to a coolsnap digital camera (Princeton Scientific Instruments, Monmouth Junction, New Jersey, USA). The fluorophore chosen for FDH location (Alexa 488) was excited using the blue filter resulting in green light emission whereas fluorophores selected for 5mTHF and FRα location were excited using the green filter given rise to red fluorescence emission. Constant exposure times (500 milliseconds) were kept within and between stains. Images were analysed for level of fluorescence using Image J 1.36 b 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" plugging. The "Threshold" plugging 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 average 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 $10\mu m^2$ 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 ($10^5$ $\mu m^2$). 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;

$$\text{Fluorescence density} \quad = \quad \frac{\text{Area positive (fluorescence above threshold) x } 10^5 \text{ } \mu m^2}{\text{Total area analysed}}$$

2.2.5.3 <u>Fluorescent dual immunostaining and sequential detection by confocal microscopy</u>

A Leica SP5 acousto-optical beam splitter confocal microscope with a 60x/0.75 numerical aperture Fluotar objective (Leica, Milton Keynes, UK) was used for sequential imaging to avoid spectral bleedthrough using the following settings: To locate FDH, a 495 nm laser line for Alexa 488 fluorophore excitation with green fluorescence emission detection at 519 nm was selected. In relation to 5mTHF and FRα identification, fluorophores Alexa 594 and Northern Lights respectively were excited using a 510 nm laser line for excitation and 590 nm for detection. Images of transmitted light were transferred to Image J software (version 1.37a; NIH, Bethesda, MA, USA). One 16 image "Z" stack was acquired per section. Sequential images were obtained, which involved exciting fluorophores on the tissue sections with only one laser at a time, and collecting fluorescent photons emitted by the excited fluorophores individually, in order to avoid cross-over of fluorescence emission of each specific channel. This approach was decided on the basis of the fluorophores broad bandwidths i.e.: 488-519 for fluorophore Alexa 488 and 510-590 for fluorophore Alexa 594 and Northern Lights.

**2.2.6 Expression of FDH mRNA in normal and hydrocephalic brain.**

2.2.6.1 <u>FDH mRNA location in normal and hydrocephalic brain using reverse transcription</u>

<u>"In situ" PCR</u>.

Reverse transcription "in situ" PCR was performed in two steps: reverse transcription and cDNA amplification using PCR. The aim was further confirmation of ALDHL1 mRNA expression in normal and abnormal brain and more importantly, the specific location of mRNA on tissue sections. A method was developed based on a modification of the technique elaborated by GJ Nuovo in 1997. The technique was optimized and adapted to our purposes using the following reagents, methodology and equipment:

**<u>Reagents:</u>**

All chemicals involved in this technique were molecular biology grade and treated with 0.1% DEPC for 12 hours, including distilled deionized water (ddw). The following buffers and solutions were prepared as described below:

-Blocking stock solution: A blocking reagent for nucleic acid hybridization and detection (Roche diagnostics, Mannheim, Germany) was solubilized in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, adjusted with Na OH to a final concentration of 10% (w/v) with shaking and heating in a microwave oven.

-Neutral buffered formalin (NBF): This buffer was prepared by dissolving 6.5 g of sodium phosphate dibasic and 4 g of sodium phosphate monobasic monohydrate in 500 ml of ddw, mix for 10 minutes, and then 100 ml of 4% paraformaldehyde were added and made up to 1 litter. pH was adjusted to 7.4 using sodium hydroxide.

-Phosphate buffer saline: This buffer was prepared by mixing 8g/L sodium chloride, 0.2 g/L potassium chloride, 1.42 g/L sodium hydrogen phosphate and 0.24 g/L potassium dihydrogen phosphate (pH 7.4).

-20 x sodium chloride-sodium citrate stock solution (SSC): 175.2 g sodium chloride and 88.2 g sodium citrate in ddw were mixed and then made up to 1 litter and solution adjusted to pH 7.0.

-Triton X-100 buffer:    0.1 M Tris-hydrochloric acid buffer (pH 7.4) containing 0.25% Triton X-100 (Sigma chemical Co. St. Louis, MO T-8787) and Igepal CA630 (Sigma, I-8896).

-RNase-Zap cleaning agent (Sigma, R2020).

-DNase I (RNase-free) and DNase buffer solution kit (New England Biolabs).

-Ribonuclease A (100µg/ml, Sigma)

-Maxima Reverse Transcriptase (Thermo scientific, EPO741) containing an RNA and DNA-dependent polymerase activity at 200U/µl (2000 U). The kit also contains 5X reverse transcription buffer and Ribolock RNase inhibitor (20 U).

-Oligo (dT)$_{18}$ primer (Thermo scientific, SO131)

- Jump-Start $^{TM}$ Taq DNA polymerase (Sigma, D4184) containing 10X reaction buffer without MgCl$_2$ which is provided separately at 25mM.

- Deoxynucleotides set solution (New England Biolab, UK limited) containing dATP, dCTP, dGTP and dTTP at 100 mM.

-Random primer Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate alkali-labile (Roche diagnostics, Mannheim, Germany).

-FDH Genbank primer. Accession number: NM_022547.1. Primers were designed using Roche ProbeFinder for rat version 2.49 ([www.rochediagnostics.qpcr.probefinder.com](www.rochediagnostics.qpcr.probefinder.com)). Primers were selected from mRNA spliced sequences as these are uniquely in mRNA and we intend to amplify exclusively cDNA after reverse transcription from mRNA. Therefore, gDNA synthesis in nuclei is in this avoided. Nevertheless, DNase treatment for inactivation of gDNA is also performed as described later in methodology (DNase digestion step).

Sense primer:  **TCGACACTCAACACTTCAGGA.**

Antisense primer:  **GGCTCCTGGGATGGGTAA**

- Anti-digoxigenin-rhodamine Fab fragments (Roche diagnostics, Mannheim, Germany).

**Methodology:**

-Preparation and pretreatment of fresh brain and liver sections.

All surgical tools and microslides used for preparation of rat tissue sections were treated with 0.1% diethylpyrocarbonate (DEPC) for 12 hours at room temperature, then autoclaved aiming to eliminate any RNase activity and potential RNA polymerase damage. All steps involved were handled with nitrile plastic gloves sprayed with RNase ZAP to remove RNase from gloves. Incubation and washes steps were performed at room temperature.

Control Sprague Dawley and hydrocephalic Texas pregnant rats were deeply anesthetized by intravenous injection with 50 mg/kg of sodium pentobarbital, and later euthanized by cervical dislocation. Rats at gestational day 19 were dissected and fetuses removed and euthanized while lay on ice for 15 minutes. Liver tissue was chosen as positive control, as

it is known is abundant in ALDHL1 protein and cDNA has been successfully isolated and characterized before in mammal liver (Min et al. 1988, Cook et al. 1991 and Hong et al. 1999). Embryonic brain, and maternal liver ("in situ" PCR tissue control), were removed quickly and frozen in isopentane cooled down by immersion on dry ice. All brains were stored at -70°C until use. Brains and liver were sectioned with a cryostat and 7µm thick sections were thaw-mounted onto the "In situ" PCR glass slides, two brain sections and one liver section (positive control) were arranged per microslide so that negative controls, positive controls and "test samples" were stained simultaneously. The sections were either store at -70 °C or transferred to the next step immediately.

The sections were fixed in NBF for 30 minutes at room temperature or overnight at 4°C, then, rinsed three times with PBS for 5 minutes each wash. After the washes, the slides were treated with 3% hydrogen peroxide in methanol for 15 minutes followed by two five minutes washes in PBS.

-DNase digestion

All sections were treated with 1U/µl of DNase I (RNase-free) solution before reverse transcription step to render gDNA template unavailable for DNA synthesis. Microslides were covered with incubation chambers RNase/ DNase free to prevent drying and incubated for 4 hours at 37°C. After incubation, the incubation chambers were removed and EDTA was included into the DNase I solution on slide at 5mM. Microslides were placed in incubator for 10 minutes at 75°C. After incubation with EDTA, slides were washed with DNase buffer for 5 minutes then rinsed in PBS-glycine (2% w/v), PBS and DEPC water for 5 minutes each. The washes were followed by treatment for 1 minute only with a graded series of ethanol (70- 100%) and air dried.

Positive control:

Rat liver tissue known to express FDH in high concentration was DNase treated, and used as positive control to assess the effectiveness of chosen FDH primers and suitability of experimental conditions for amplification of mRNA/cDNA using "In situ" PCR technique. One liver tissue section was placed per microslide along with negative control and "test section".

Negative control:

One section per microslide was subjected to DNase treatment, and reverse transcription but, on this occasion, reverse primer or forward primer was omitted in the PCR master mix to demonstrate signal is due to amplification of cDNA by "In situ" PCR. For this reason, signal is not expected in negative controls.

Test sections:

One section per microslide is chosen as "test sections", therefore, DNase treatment and RTPCR reagents are added to this section for reverse transcription of specific FDH mRNA to cDNA first, and later cDNA amplification with FDH primers. Positive staining  (FDH mRNA/cDNA-Rhodamine labelled) was expected in cytoplasm of cells exclusively in these sections.  This is due to the fact that all DNA, other than cDNA (mRNA), is inactivated by DNase treatment previously to reverse transcription. Furthermore and as aforementioned,  gDNA synthesis is avoided by chosen primers from unique sequences of mRNA that are not present in gDNA as they occur after mRNA maturation and splitting, resulting in mRNA sequences with non-contiguous regions in the DNA.

-Reverse transcription. First step RT in situ PCR.

All reagents and microcentrifuge tubes were placed on ice, and a specific lab area was designated to perform the reaction. In a sterile microcentrifuge tube, the following reagents were assembled to cover just <u>one test section</u>:

| | |
|---|---|
| Primer oligo (dt) $_{15}$ at 500µg/ml (stock) | 3µl |
| dNTPs (each nucleotide at 10 mM) | 3µl |
| RT maxima buffer (x1) | 12µl |
| RNase inhibitor | 1.5µl |
| RT maxima at 200 U/µl (stock) | 1µl |
| H$_2$O | 60µl |

50µl of reaction mixture was added per test section. To prevent sample dehydration during RT reaction, an incubation chamber was anchored to the slide before incubation on a thermal cycle block set at 50°C for 30 minutes followed by 85°C for 5 minutes. DEPC water was added to sections chosen on slide as controls.

-First SSC treatment.

After the reverse transcription step, all sections on the slide were washed twice with SSC buffer at room temperature for 10 minutes, followed by a final wash at 45°C for 10 minutes, and they were then air dried.  CDNA amplification by PCR was carried out straight after SSC treatment.

-PCR. Second step RT in situ PCR.

In a sterile microcentrifuge tube, the following reagents were added in the following order to prepare 130 µl of reaction mixture, enough to cover one slide i.e.: three sections.

| | |
|---|---|
| Forward primer | 5.2 µl at 0.5 µM (final concentration) |
| Reverse primer | 5.2 µl at 0.5 µM (final concentration) |
| dATP | 2.6 µl at 200µM (final concentration) |
| dGTP | 2.6 µl at 200µM (final concentration) |
| dCTP | 2.6 µl at 200µM (final concentration) |
| dTTP | 1.56 µl at 100µM (final concentration) |
| DIG-11- dUTP | 1.56 µl at 1mM   (stock) |
| Mg Cl$_2$ | 20.8 µl 25 mM (stock) |
| Blocking solution | 13 µl (10% w/v) |
| PCR buffer (x10) | 13 µl |
| Jump start enzyme | 30.12µl (60 units/slide) |
| H$_2$O | to 130 µl |

Each slide is coated with 130 µl of PCR reaction mixture, covered with an incubation chamber, and finally placed on thermocycler set up to perform the thermal cycle series indicated on table 2.1below:

| Cycle number | Denaturation | Annealing | Polymerization |
|---|---|---|---|
| 1 | 1 min at 94°C | | |
| 35 | 30 sec at 94°C | 1 min at 55°C | 1 min at 72°C |
| 1 | | | 1 min at 72 °C |

**Table 2.1. Thermocycler parameters used for "in situ" PCR i.e.: cDNA amplification.**

The newly formed cDNA was to be detected using the probe dig-11-dUTP after RT-PCR rather than by directed labelling.

-Second SSC treatment.

Slides were subjected to high stringency conditions by washing with 0.2 x SSC twice at 60C° for 10 minutes each, followed by rinsing in PBS for 5 minutes.

Equipment:

-A cryostat MicronHM 500 (Micron, Heidelberg, Germany) was used for obtaining tissue sections.

-A G-Storm GSI thermocycler (Labtech International/Tecan Ltd. Reading. UK) and CoverWell ™ incubation chambers - RNase/ DNase free - were used, as well as microslides, superfrost ultraplus (VWR International Ltd.), for mRNA reverse transcription to cDNA and its amplification on brain tissue sections attached to microslides.

-A Leica DMLB fluorescence microscope coupled to a coolsnap digital camera (Princeton Scientific Instruments, Monmouth Junction, New Jersey, USA) was used to take images of transmitted light, as explained in paragraph 2.2.5.2. All images were transferred to Image J software (version 1.37a; NIH, Bethesda, MA, USA).

-A Leica SP5 acousto-optical beam splitter confocal microscope with a 60x/0.75 numerical aperture Fluotar objective (Leica, Milton Keynes, UK) was used for simultaneous imaging of DAPI and Rhodamine. 405/450 excitation/emission laser lines for DAPI and 510/590 excitation/emission laser lines for detection of FDH mRNA expression were used respectively. Images of transmitted light were transferred to Image J software (version 1.37a; NIH, Bethesda, MA, USA). One 16 image "Z" stack was acquired per section.

2.2.6.2 <u>FDH mRNA quantification in cerebral cortex tissue using real time quantitative</u>
<u>PCR.</u>

Real-time quantitative PCR (RTqPCR) was used to measure FDH mRNA expression in normal and hydrocephalic brain. Six normal and five hydrocephalic samples were analysed in parallel (each sample representing an individual cerebral cortex). Total RNA was extracted from whole fetal cerebral cortex using TRIzol reagent following the manufacturer's instructions (Invitrogen, UK). To synthesize cDNA, 2µg RNA was reverse transcribed using oligo $(dT)_{15}$ primers (Promega, UK) and Maxima Reverse Transcriptase (Thermo Scientific, UK). Rat liver total RNA, also extracted with TRIzol reagent in the same conditions as cerebral cortex, served as positive control. Omission of reverse transcriptase was used as negative control. cDNA at 400ng/µl was amplified using SYBR Green Jump Start master mix following the company's instructions (SIGMA). cDNA integrity was confirmed by b-actin amplification as described previously. Amplification of tyrosine 3-monooxigenase/tryptophan 5-monooxygenase activation protein (YWHAZ) mRNA (Table 2.2), a stable housekeeping 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 Gen-Bank sequences using Roche ProbeFinder for rat version 2.49 (www.rochediagnostics.qpcr.probefinder.com). mRNA expression was quantified using Step-One AB Aplied Biosystems with normalization to positive control (liver) as internal calibrator. 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 2.2) 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

Aplied Biosystems software version 2.2 instructions.

| Genes (rat) | Accession number | $T_a$ (°C) | Sense primer | Antisense primer |
|---|---|---|---|---|
| FDH | NM_022547.1 | 55 | TCGACACTCAAC ACTTCAGGA | GGCTCC TGGGA TGGGTAA |
| BACTIN | NM_031144.2 | 60 | CCCGCGAGTACA CCTTCT | CGTCATCCATGG CGAACT |
| YWHAZ | NM_01135699.1 | 55 | CTACCGCTACTT GGCTGAGG | TGTGACTGGTC CACAATTCC |

**Table 2.2. Gene sequences and annealing temperatures ($T_a$) for primers targeting FDH/ALDH1L1 and housekeeping genes.**

## 2.3 RESULTS

### 2.3.1 5mTHF in combination with FDH is detrimental for cell growth.

2.3.1.1 Decreasing concentration of 5mTHF plus constant FDH
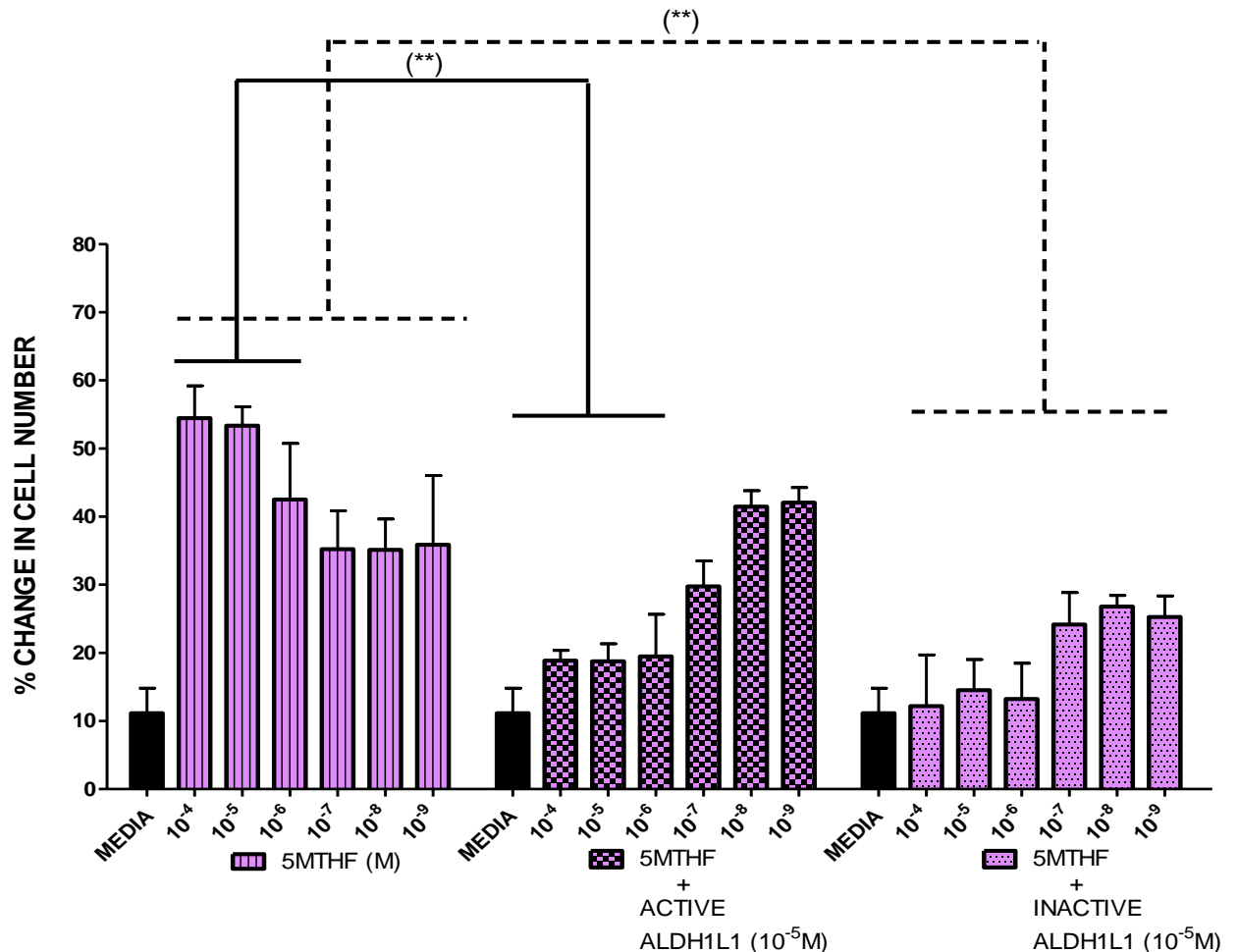


**Figure 2.3. Effect of decreasing 5mTHF and constant active and inactive FDH concentration on cell proliferation. MTHF alone (line pattern), 5mTHF + active FDH (square pattern) and 5mTHF + inactive FDH (dot pattern). Primary cortical cells from E19 Sprague- Dawley foetuses were incubated with the metabolite at $10^{-4}$ - $10^{-9}$M, as illustrated in the graph, in the absence and presence of FDH at $10^{-5}$ M. Cell bioavailability was measured using a fluorescence-based assay, as described in material and methods (2.2.1). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of variance (two-ways ANOVA) with Bonferroni post hoc test revealed significant differences in the indicated paired samples. Results are shown as: \*\*$p < 0.01$ for the effect on proliferation of 5mTHF alone versus 5mTHF in combination with FDH.**

In order to determine the effect of constant FDH and decreasing 5mTHF concentration on cell growth, the metabolite was added to neurobasal media at concentration range $10^{-4}$- $10^{-9}$ M, with and without active and inactive FDH at $10^{-5}$ M. Control experiments showed that E19 cortical cells cultured in neurobasal media increased overall in cell number above media control levels (no folates) after 24 hours (Figure 2.3). The average percentage increases were of $42.7 \pm 6$, $28.38 \pm 3.08$ and $13.84 \pm 4.34$ for media containing 5mTHF, 5mTHF plus active FDH, and 5mTHF plus inactive FDH respectively. Media with 5mTHF alone at a range of concentration $10^{-4} - 10^{-6}$ M caused a percentage increase in cell number of $54.43 \pm 4.7$, $53.34 \pm 2.7$ and $42.50 \pm 8.2$. These data show that cell proliferation was directly proportional to the concentration of 5mTHF in the media. By contrast, in general, the presence of FDH in combination with 5mTHF showed a clearly negative effect on cell growth with, both active and inactive protein. Thus, the addition of active FDH caused significant percentage decreases in cell number of $35.59 \pm 1.31$, $34.57 \pm 2.54$ and $23.02 \pm 6.17$ when compared to media containing 5mTHF alone at $10^{-4}$, $10^{-5}$ and $10^{-6}$ M respectively, ($p < 0.01$). Moreover, the inhibitive effect of FDH on cell growth was null at 5mTHF $10^{-8}$ and $10^{-9}$ M, with percentage increase values of $6.16 \pm 2.34$ and $6.33 \pm 2.22$ respectively in the presence of active FDH. However, these results were not significant ($p>0.05$)

In relation to inactive ALDHL1, a generalized negative effect on growth at all 5mTHF concentrations occurred, with significant percentage decreases ($p < 0.01$) as follows: $42.23 \pm 7.51$, $38.81 \pm 4.48$, $29.26 \pm 5.20$, $11.08 \pm 4.71$, $8.32 \pm 1.61$ and $10.60 \pm 3.07$.

2.3.1.2 <u>Decreasing concentration of FDH plus constant 5mTHF</u>

Past Western blotting experiments allowed relative quantification of FDH in CSF of normal rats, revealing FDH in CSF is present at $10^{-5}$M (Cain et al. 2009). By contrast, a negligible signal was detected in hydrocephalic CSF. The experiments illustrated in figure 2.3 show some cell growth in the presence of ALDHL1 at $10^{-5}$M, along with 5mTHF at $10^{-8}$M, either with active or inactive FDH. These values were considered to further study the cell response to 5mTHF in combination with FDH but, on this occasion using FDH at concentration range $10 - 0.31 \times 10^{-5}$ M and constant 5mTHF at $10^{-8}$M. Results from this experiment are shown in figure 2.4. This graph indicates that cortical cells cultured in neurobasal media with 5MTHF had a generalized increase in cell proliferation above media control levels following 24 hours growth at all concentrations. The average percentage increase was $34.16 \pm 2.26$ with 5MTHF alone and $16.46 \pm 3.32$ in the presence of 5mTHF plus active FDH. Cell proliferation was also slightly encouraged with 5mTHF plus inactive FDH, with an average percentage increase of $20.91 \pm 4.03$. In conclusion, 5MTHF alone without FDH led to the highest percentage increase in cell number.
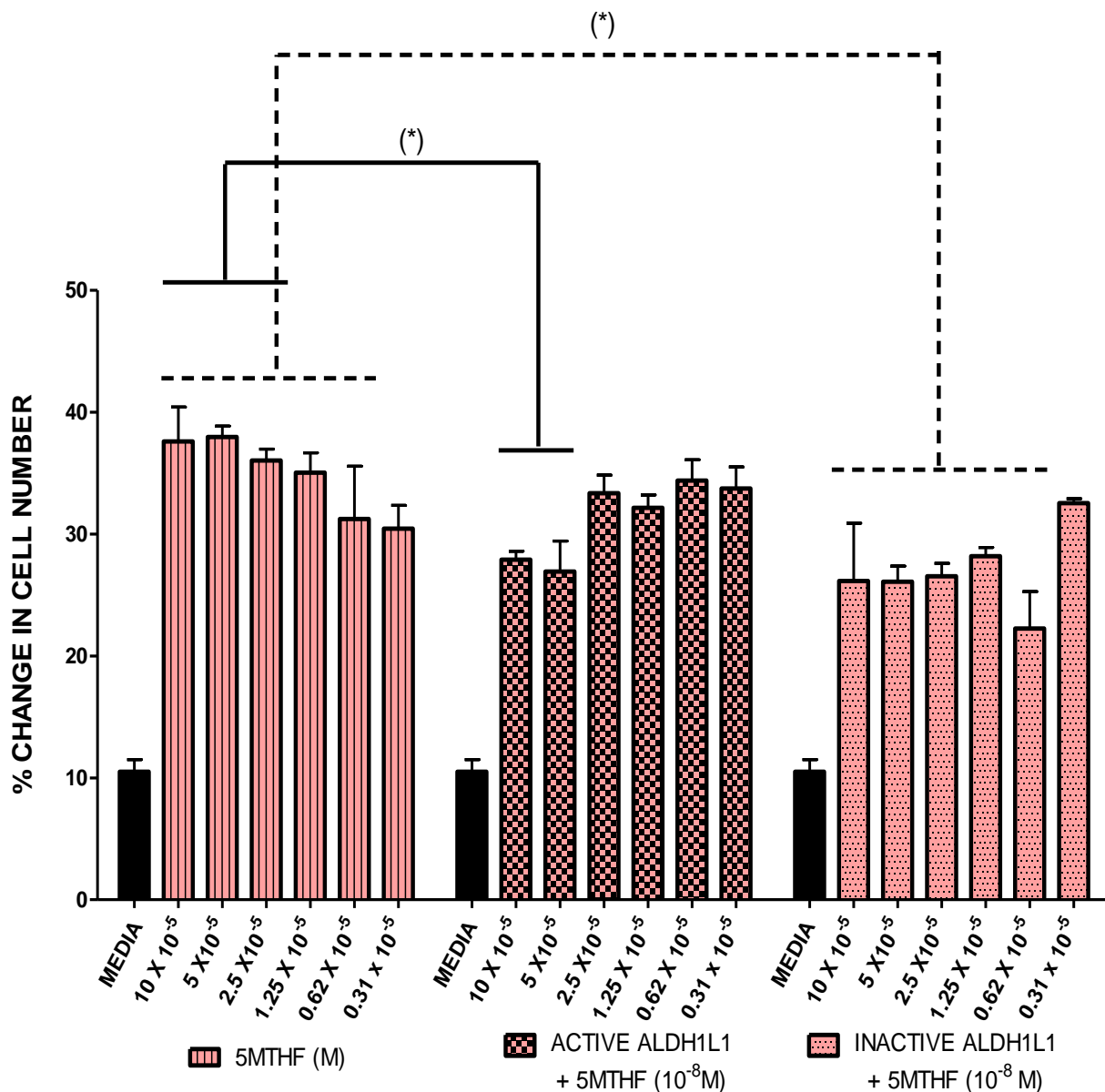
**Figure 2.4. Effect on cell proliferation of decreasing 5mTHF alone, decreasing active FDH plus constant 5mTHF and decreasing inactive FDH plus constant 5mTHF. 5mTHF alone (line pattern), 5mTHF + active FDH (square pattern) and 5mTHF + inactive FDH (dot pattern). Cell bioavailability was measured using a fluorescence-based assay as described in material and methods (2.2.1). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of variance (two-ways ANOVA) with Bonferroni post hoc test revealed significant differences in the indicated paired samples. Results are shown as: *p < 0.05 for the effect on proliferation of decreasing 5mTHF alone versus constant 5mTHF in combination with decreasing active and inactive FDH.**

Figure 2.4 also shows how 5mTHF at constant concentration in the presence of active FDH at 10 x $10^{-5}$ and 5 x $10^{-5}$ M reduced cell growth significantly with percentage decreases of 9.73 ± 0.75 and 11.04 ± 2.58 respectively (*p<0.05). However, the decrease in growth was less pronounced at FDH concentration of 2.5 x $10^{-5}$ and 1.25 x $10^{-5}$, with percentage decreases of 2.71 ± 3.44 and 2.93 ± 1.67. Finally, a relative percentage increase occurred at FDH concentration of 0.62 x $10^{-5}$ and 0.31 x $10^{-5}$ M with percentage increases values of 3.15 ± 1.69 and 3.33 ± 1.76.

In relation to inactive FDH, there was a generalized decrease in cell number at concentration range 10-0.62 x $10^{-5}$ M with percentage decreases of 11.45 ± 4.73, 11.86 ± 1.27, 9.52 ±1.06, 6.85 ± 0.70 and 9.12 ± 3.04 (*p<0.05). Similar to the results obtained from media treated with active FDH, a relative percentage increase of 2.14 ± 0.35 was obtained at 0.31 x $10^{-5}$ M.

In summary, the positive effect on cell growth of 5mTHF can be perturbed by adding FDH to the media, and this effect is more intense in the presence of inactive FDH than active FDH. Importantly, FDH's inhibitory effect on cell growth is observed at high concentration in the range 10-5 x $10^{-5}$ M, suggesting a concentration effect of FDH on cell growth.

## 2.3.2 Cell growth is not inhibited with folic acid in the presence of FDH
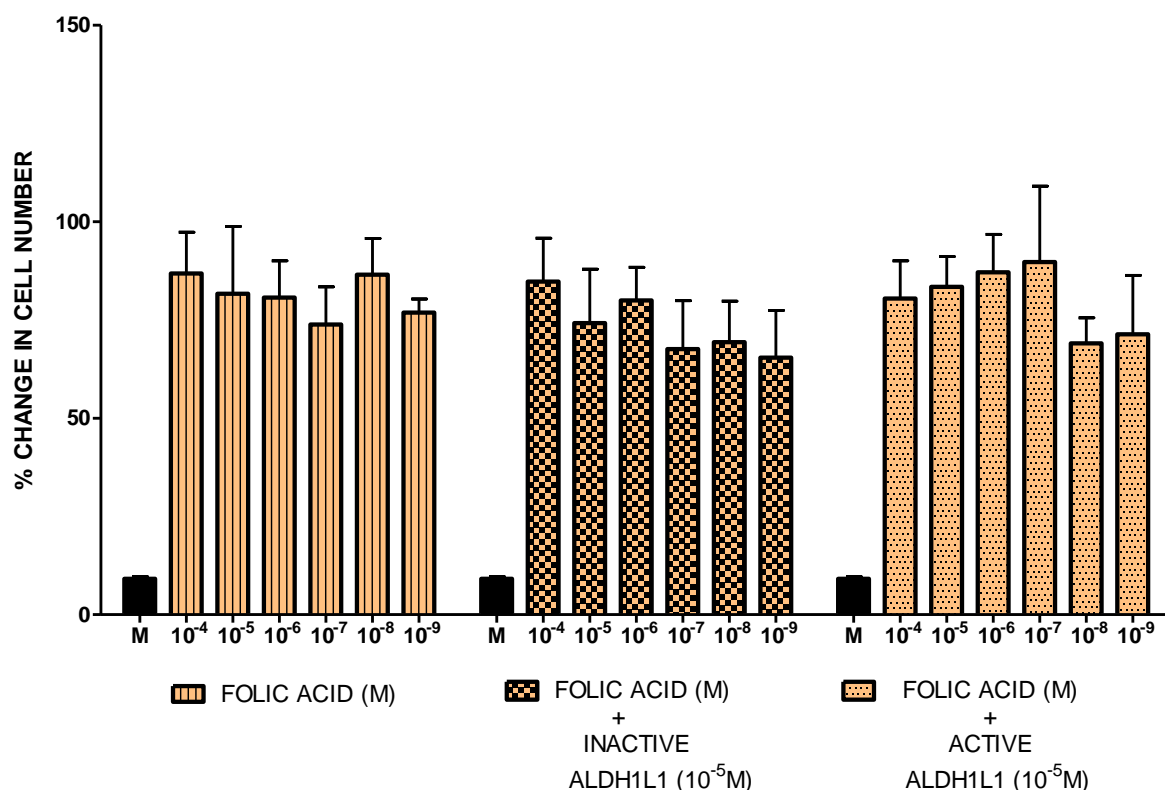


**Figure 2.5. Effect on cell proliferation of folic acid alone (line pattern), folic acid plus inactive ALDHL1 (square pattern) and folic acid plus active FDH (dot pattern) all at decreasing concentrations. Cell bioavailability was measured using a fluorescence-based assay as described in material and methods (2.2.1). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6).**

Folic acid is an artificial and stable folate commonly found within commercial media. The media control used throughout all experiments did not contain folic acid. Thus, folic acid was added to neurobasal media, and its effect on cell division assessed by comparison with media control in the presence and absence of inactive and active FDH. Figure 2.5 shows that cells in all conditions presented an average percentage increase above media control levels of 82.43 ± 7.75. On the other hand, media containing folic acid alone versus media with the folate in combination with either inactive or active FDH showed some small cell growth when FDH was present at specific concentrations. Thus, the average percentage

decreases due to FDH presence were 8.86 ±3.57 with inactive FDH and 8.84 ± 2.54 with active FDH. In conclusion, FDH did not perturb cell growth in the presence of folic acid.

### 2.3.3 **Commercial and unstable THF does not promote growth with or without FDH.**
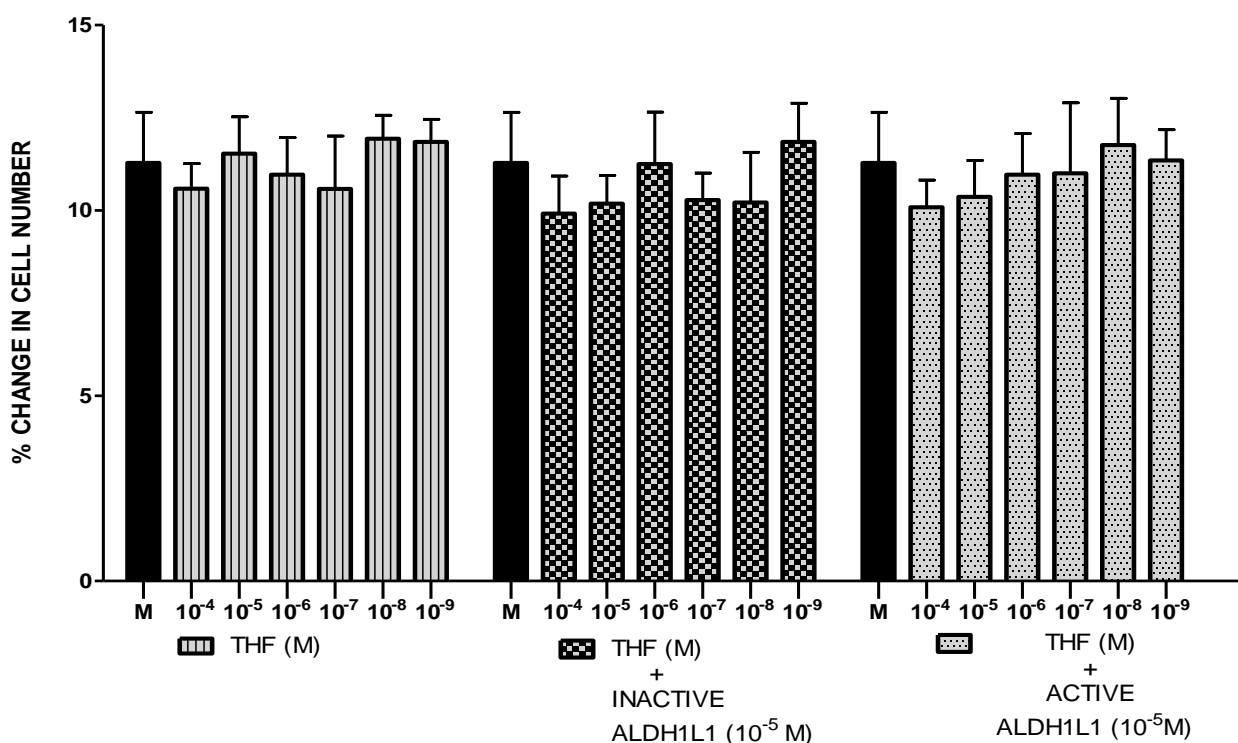


**Figure 2.6. Effect on cell proliferation of THF alone (line pattern), THF plus inactive ALDHL1 (square pattern) and THF plus active FDH (dot pattern) all at decreasing concentrations. Cell bioavailability was measured using a fluorescence-based assay as described in material and methods (2.2.1). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6).**

THF is the product of FDH enzymatic reaction, and the effect of both molecules on cell division was determined by adding them to neurobasal media. Thus, THF alone, THF in combination with FDH and finally THF with active FDH were tested on cell growth. Cell growth was nulled or negligible in all conditions (Figure 2.6).

2.3.4 **DAPI staining of cell cultures treated with folates and FDH**

DAPI staining was carried out in parallel to folates effect on cell proliferation, which was described in section 2.2.2. DAPI staining results in the presence of specific folates with and without FDH are shown in Figure 2.7 below:
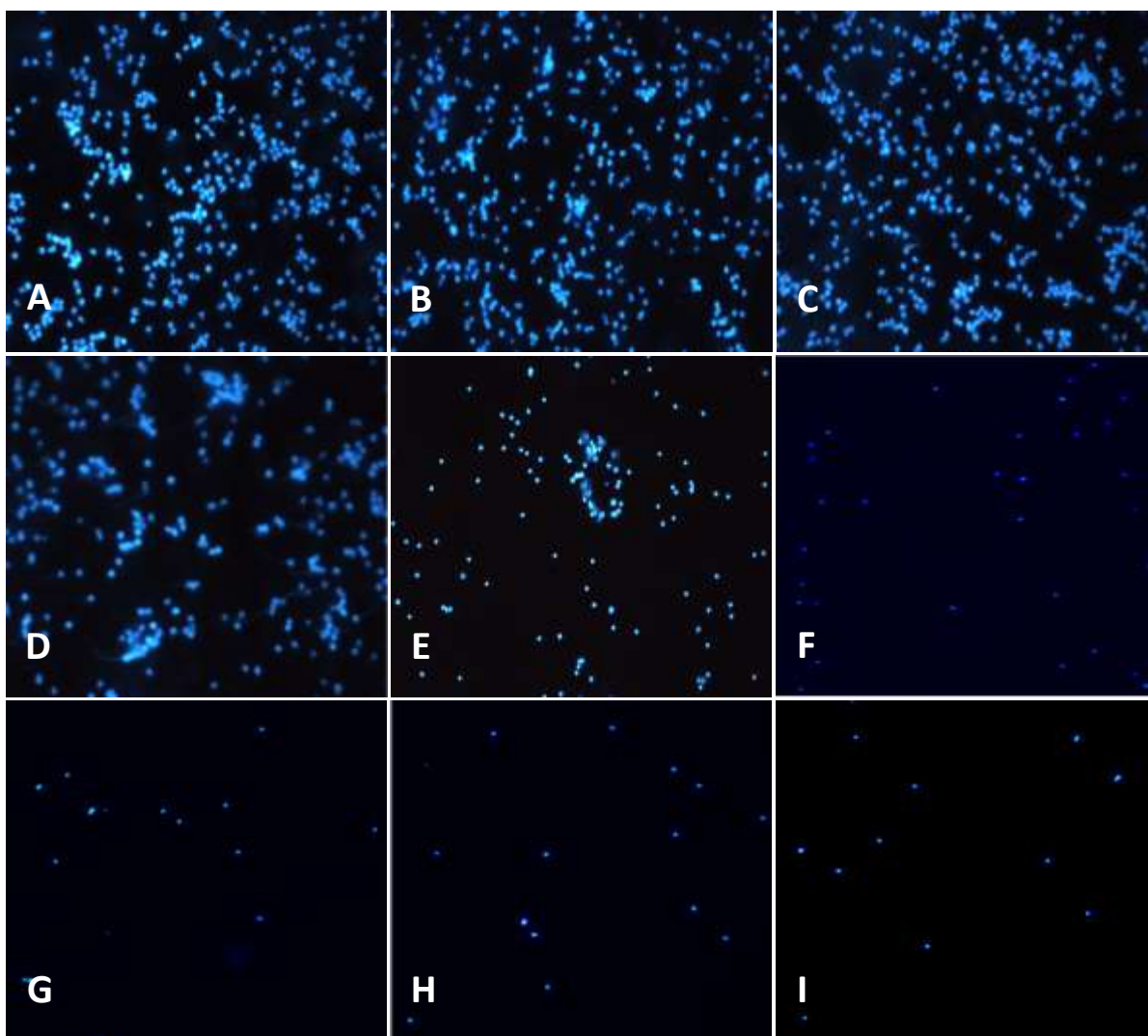


**Figure 2.7. DAPI nuclei staining snapshots of primary cell cultures in the presence of different types of folates (10 $^{-5}$ M) with and without FDH (10 $^{-5}$ M) as follows: folic acid (A), folic acid + inactive FDH (B), folic acid + active FDH (C), 5mTHF (D), 5mTHF + inactive FDH (E), 5mTHF + active FDH (F), THF (G), THF + inactive FDH and THF (H) + active FDH (I), (x20) magnification.**

These results are in accordance with previous outcomes obtained from studies of folates' effects on cell proliferation (2.3.1, 2.3.2 and 2.3.3 sections) and visually demonstrate how

folic acid in the presence and absence of FDH exacerbates cell proliferation (Figure 2.7, A, B and C). In relation to DAPI staining of cells grown with 5mTHF, these results visually confirm how this folate potentiates growth when present alone in the media (Figure 2.7, D). Interestingly, this positive effect is compromised by the addition of inactive FDH (Figure 2.7, E) and active FDH (Figure 2.7, F). Finally, THF alone (Figure 2.7, G) and either in combination with inactive FDH (Figure 2.7, H) or active FDH (Figure 2.7, I) do not have any impact on cell growth.

### 2.3.5 THF conjugated to immunoglobulins (IgG) has a positive effect on cell growth.

2.3.5.1 <u>DAPI staining of cell culture treated with IgG-THF</u>.

DAPI staining snapshots obtained from cell culture treated with IgG-THF are shown in figure 2.8 and DAPI fluorescent measurements are illustrated in figure 2.9. These results demonstrate that conjugated THF has a pronounced positive effect on cell growth in contrast to growth with commercially unstable THF alone or with no folate at all.
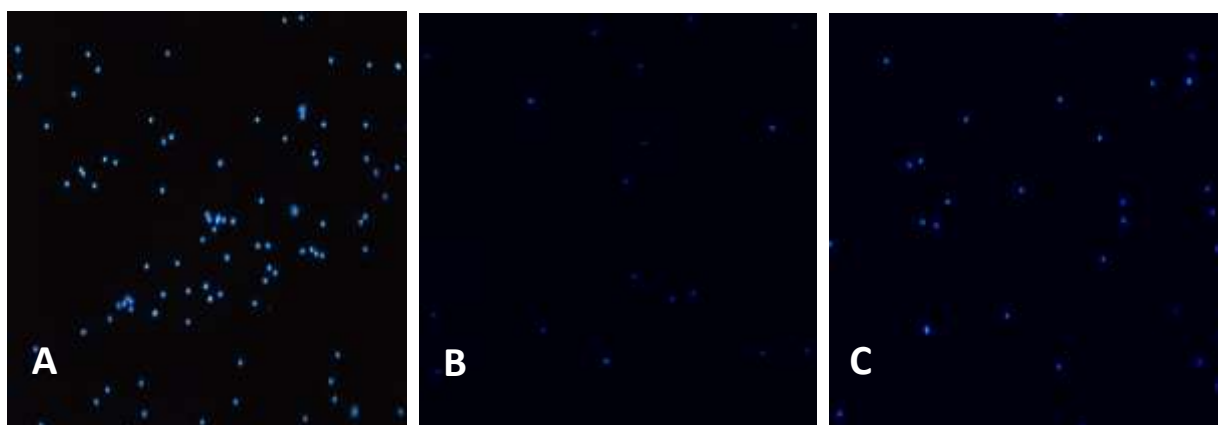


**Figure 2.8 shows DAPI nuclei staining snapshots of primary cell cultures in the presence of IgG-THF at $10^{-5}$ M (A), THF at $10^{-5}$ M (B) and media control (x 20 magnification).**

Accordingly, a 12.8 % increase in cell number occurred when cells were cultured in media

with IgG-THF conjugates. This contrasts with minimal cell growth in cultures treated with

media containing THF only.
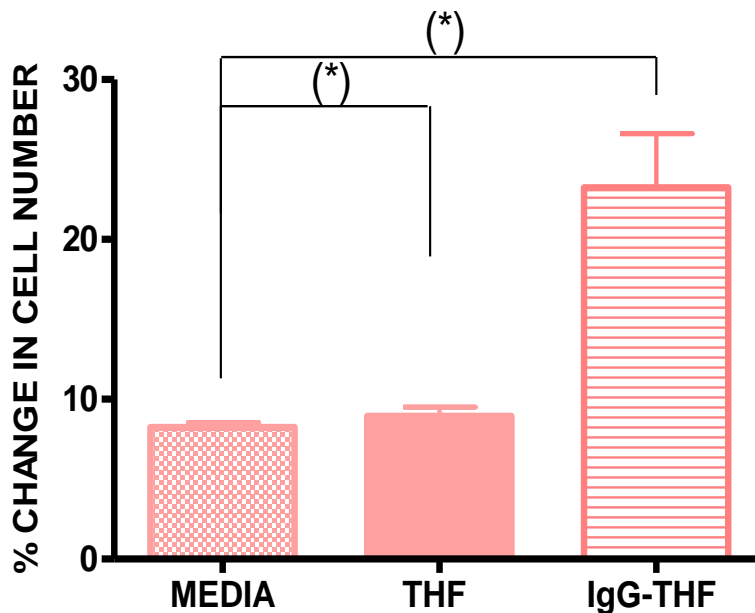


**Figure 2.9 shows inhibitory effect of THF as opposed to IgG-THF's positive effect on cell growth of primary cerebral cortex cells at embryonic day 18. Cell growth was determined by measuring fluorescence due to DAPI-dsDNA complex formation using a fluorimeter set up at 358 nm excitation and 461 emission wavelengths. Data is presented as mean fluorescence readings ± SEM (n=3). Analysis of data with Kruskas-Wallis test and post-test Dunn's multiple column comparison revealed significant differences. Results are indicated as *p<0.05.**

2.3.5.2 <u>IgG-THF fluorescent tracking demonstrates THF is taken in by cerebral cortex cells</u>.
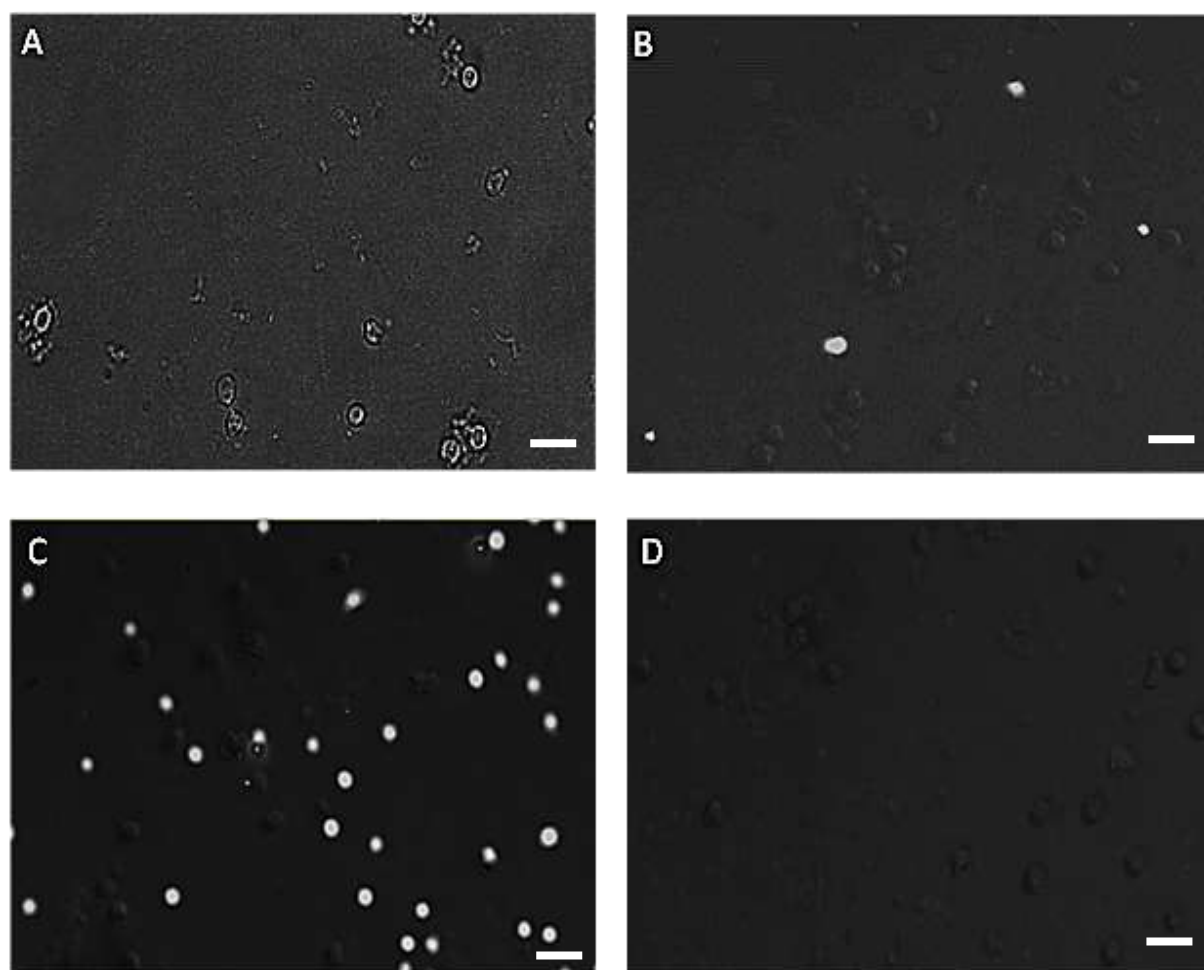


**Figure 2.10 Uptake of fluorescent THF-labelled IgG at 10** $^{-5}$ **M by cerebral cortex cells. Fluorescence microscope snapshots (x60) of cerebral cortex cells incubated with IgG-Cy3-THF (25µg/ml) were taken after 12 hours (A), 16 hours (B) and 24 hours (C). Cells incubated with IgG labelled to the same extent with Cy3 but lacking any conjugated THF are shown in D (negative control), (Scale bar = 100 µm),**

The effect of THF in media deprived of any other folates and FDH was studied using THF conjugated to a monoclonal fluorescent THF antibody or immunoglobulin G type 1. Fluorescence inside cells was detected using an inverted fluorescence microscope which was set up at a maximum absorbance suitable for Cy3 (670 nm). The fluorescence signal was observed on the cell periphery after 12 hours growth when THF-conjugated Cy3 – labelled IgG was present in the media (Figure 2.10, A). THF entered the cells gradually over a period of 24 hours as cells divided (B and C). Cells growing in media without THF

but containing Cy3 –labelled IgG did not show any fluorescence, meaning that Cy3 labelled IgG was not accumulated by these cells. This suggests that attachment of fluorescent IgG to the folate promotes the uptake of THF-conjugated Cy3 –fluorescent IgG, as shown in photos A, B and C. These results showed that cerebral cortex cells were able to divide without the presence of FDH, and revealed that cell division can occur with THF only in the media. However, these results contradicted previous data (Figure 2.6) which suggested that unconjugated THF in cell culture leads to negligible growth. This finding emphasizes the importance of using stable THF in this type of cell culture study.

2.3.5.3 <u>Bioavailability studies suggest conjugated THF has a positive effect on cell growth</u>.
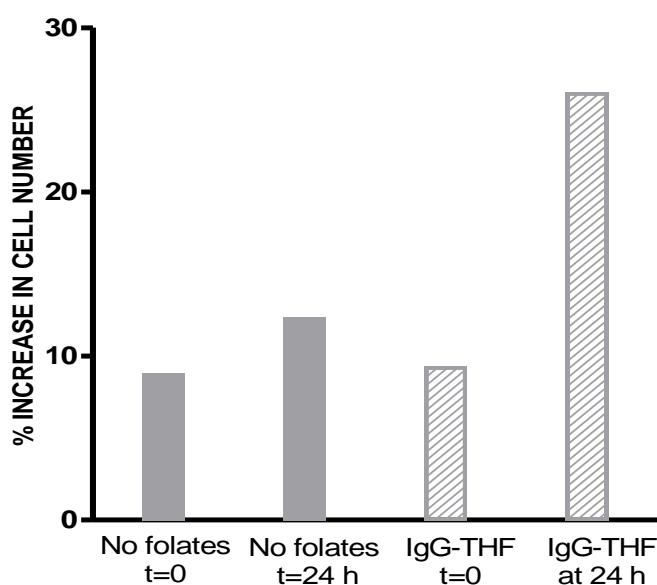


**Figure 2.11 Effect on cell proliferation of THF (line pattern) versus no folates (plain pattern) at starting point (zero growth; t=0) and after 24 hours (t=24 h). Fluorescence was measured using a fluorescent PrestoBlue cell viability reagent kit as described in material and methods (2.2.3.3). Data are presented as percentage increases in cell number obtained after normalization of relative fluorescence indexes before and after 24 hours growth.**

Cell bioavailability was studied at the start (t=0) and at the end of the experiment (t=24) in the presence and absence of IgG-THF (Figure 2.11 above). Cell growth increase (14.4%) was obtained from cells cultured in the presence of IgG-THF after 24 hours, compared to growth with IgG-THF at the start of the experiment (t=0). In contrast, minimal percentage increases occurred with cells grown in media without folates at t=0 and t=24h. These results indicate a positive effect on cell growth by THF-IgG after 24 hours, and provide evidence about the suitability of immunoglobulins for the stabilization of oxygen sensitive vitamins like THF.

2.3.6 **Negative FDH effect on cell growth depends on folate presence in the media**.
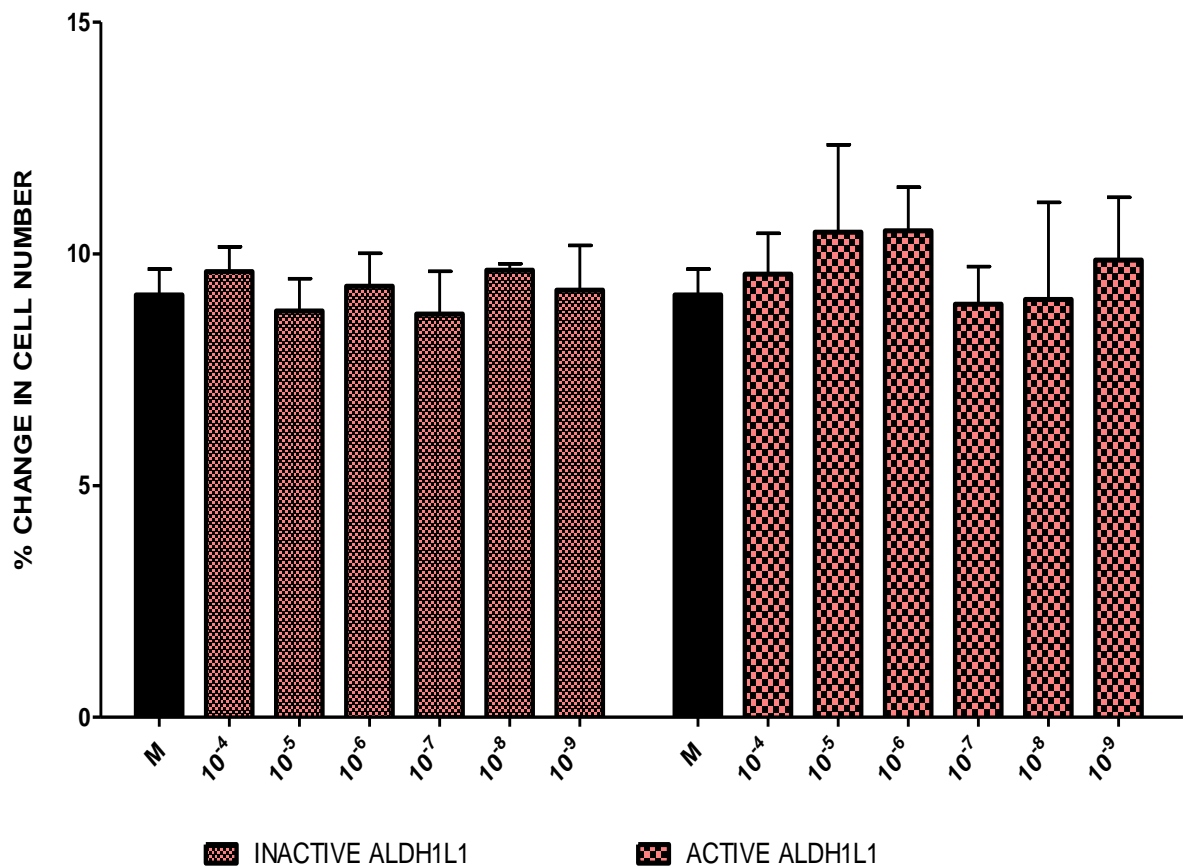


**Figure 2.12. Effect on cell proliferation of active (small squares) and inactive ALDH1L1 (FDH) (large squares) Proliferation was measured using a fluorescence-based assay, as described in material and methods (2.2.1). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of data with Kruskas-Wallis test and post-test Dunn's multiple column comparison revealed there was no effect of FDH on cell growth.**

To assess the role of active and inactive FDH on the cerebral cortex, the folate binding protein was added to the media without any folates. Cortical cells cannot survive without folates and as expected, addition of FDH alone to neurobasal media led to a significant minimal average percentage increase of 0.34 ± 1. 28 when compared to the media control.

### 2.3.7 **FDH shows affinity for 5mTHF.**

2.3.7.1 <u>Standard curves parallelism between non-commercial and commercial FDH is
confirmed using a commercial FDH ELISA prior to development of an "in house" ELISA
for detection of 5mTHF binding to FDH</u>
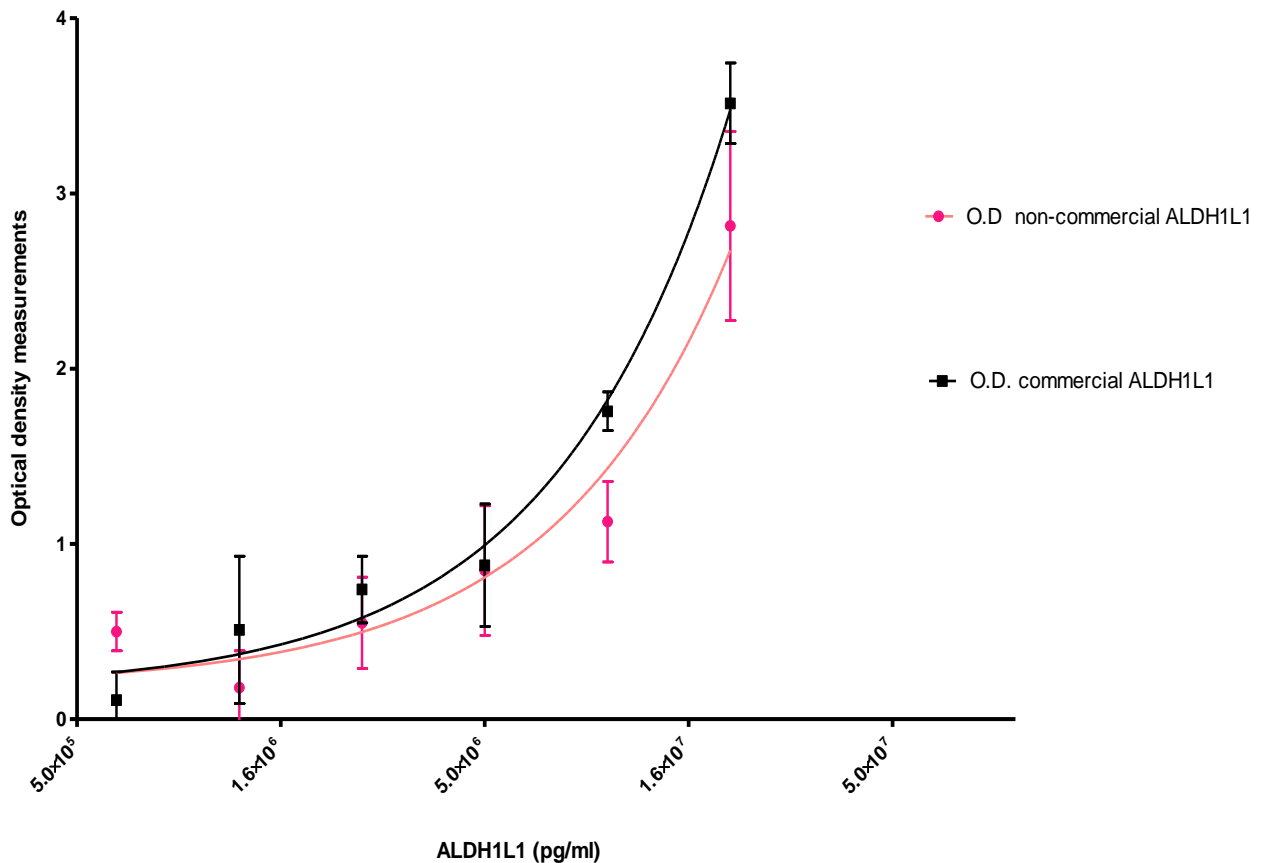


**Figure 2.13.** <u>**Commercial and non-commercial (FDH) standard curves**</u>**. Standard
curves were produced by plotting optical density measurements versus commercial
kit standard FDH (black line) and non-commercial standard protein (pink line).
Curves fits were compared using a non-parametric paired Wilcoxon rank test
(p<0.05).**

Standard curves were created in order to ensure FDH was bound to the ELISA kit solid

phase using first the kit's FDH standard protein and later the non-commercial FDH    (Dr.

Krupenco's) which was used in all previous "in vitro" experiments. The aim was to

demonstrate that non-commercial FDH behaves similarly to commercial protein. The kits

reagents and optimized experimental conditions recommended by the company were used to produce the standard curves. The kit's detection system, based on a colorimetric reaction (HRP-Avidin-TMB), allowed optical density measurements of FDH concentration. Optical density values were plotted versus FDH concentration to obtain the standard curves (Figure 2.13). The standard curves described a logarithmic trend and run in parallel, demonstrating that curve medians were not significantly different ($p < 0.05$). In other words, the curves' paired values were significantly correlated, and therefore similar binding properties of commercial and non-commercial ALDHL1 to the monoclonal FDH antibody on the solid phase were established. These experiments helped to confirm the conditions needed to progress further towards the development of a fluorescent ELISA immunoassay that allows quantification of the potential binding between FDH and 5mTHF. An FDH standard curve was always run simultaneously to the fluorescent immunoassay to confirm FDH attachment to solid phase.

2.3.7.2 "In house ELISA" using commercial FDH ELISA and fluorescent anti-5mTHF antibody shows FDH affinity for 5mTHF.

To assess whether there is affinity of FDH for 5mTHF, and therefore FDH-5mTHF complexes attached to the ELISA solid phase, plates were first coated with FDH, followed by incubation with 5mTHF. Equal concentrations of FDH and 5mTHF were added to the wells in order to achieve concomitant double serial dilutions for both molecules. Estimation of amount of 5mTHF bound to FDH was achieved by using an "in house" fluorescent 5mTHF antibody. There was a relative linear relationship between fluorescence and 5mTHF concentration, with $r^2 = 0.74$, demonstrating 5mTHF binds to FDH. Figure 2.14 below:
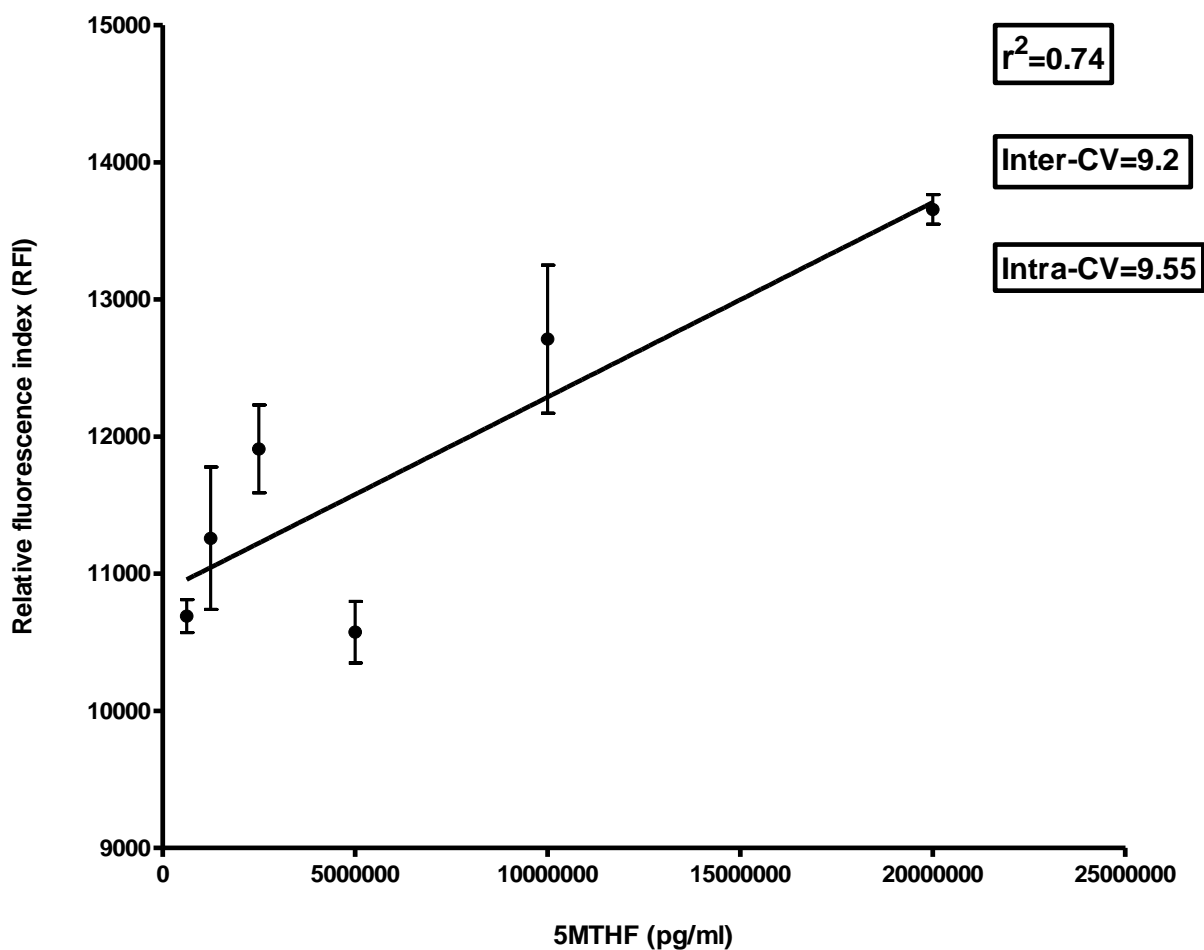
**Figure 2.14. "In house" Fluorescent ELISA assay.** **Study of potential FDH binding to 5mTHF was carried out using an "in house" fluorescent immunoassay. Fluorescent values were plotted versus 5mTHF concentration. A relative linear relationship with an $r^2=0.74$ demonstrated FDH affinity for 5mTHF. The intervariation and intravariation coefficient of the assay was 9.2 and 9.55 respectively. N=3**

Fluorescence values were included only if their mean exceeded by more than two standard deviations the mean optical density given by the negative controls (buffer only), thereby establishing a cut-off value. Fluorescence values obtained from 5mTHF dilutions were all $\geq$ than the cut-off value. Cut-0ff= Mean OD (–C) + 2SD= 136.

To study the variability of the method within the assay and between assays, intravariation and intervariation coefficients were calculated respectively. Top, middle and low standard curve values from different experiments were used to calculate both variation coefficients using the following formula: Variation coefficient=SDx100/mean.

Intravariation coefficient (intra-CV) was 9.2 and intervariation coefficient (inter-CV) was 9.55. Finally, the graph end fluorescence measurement shows a value closed to the penultimate fluorescence reading, indicating that the assay may reach saturation at concentrations of 5mTHF higher than $2x10^7$ pg/ml.

2.3.8. **FDH protein colocalizes with 5mTHF and FRα**

Representative photomicrographs of FDH and 5mTHF immunohistochemical localization in cerebral cortex (CC) and choroid plexus (CP) in normal and hydrocephalic brain (Figure 2.15 & Figure 2.16)
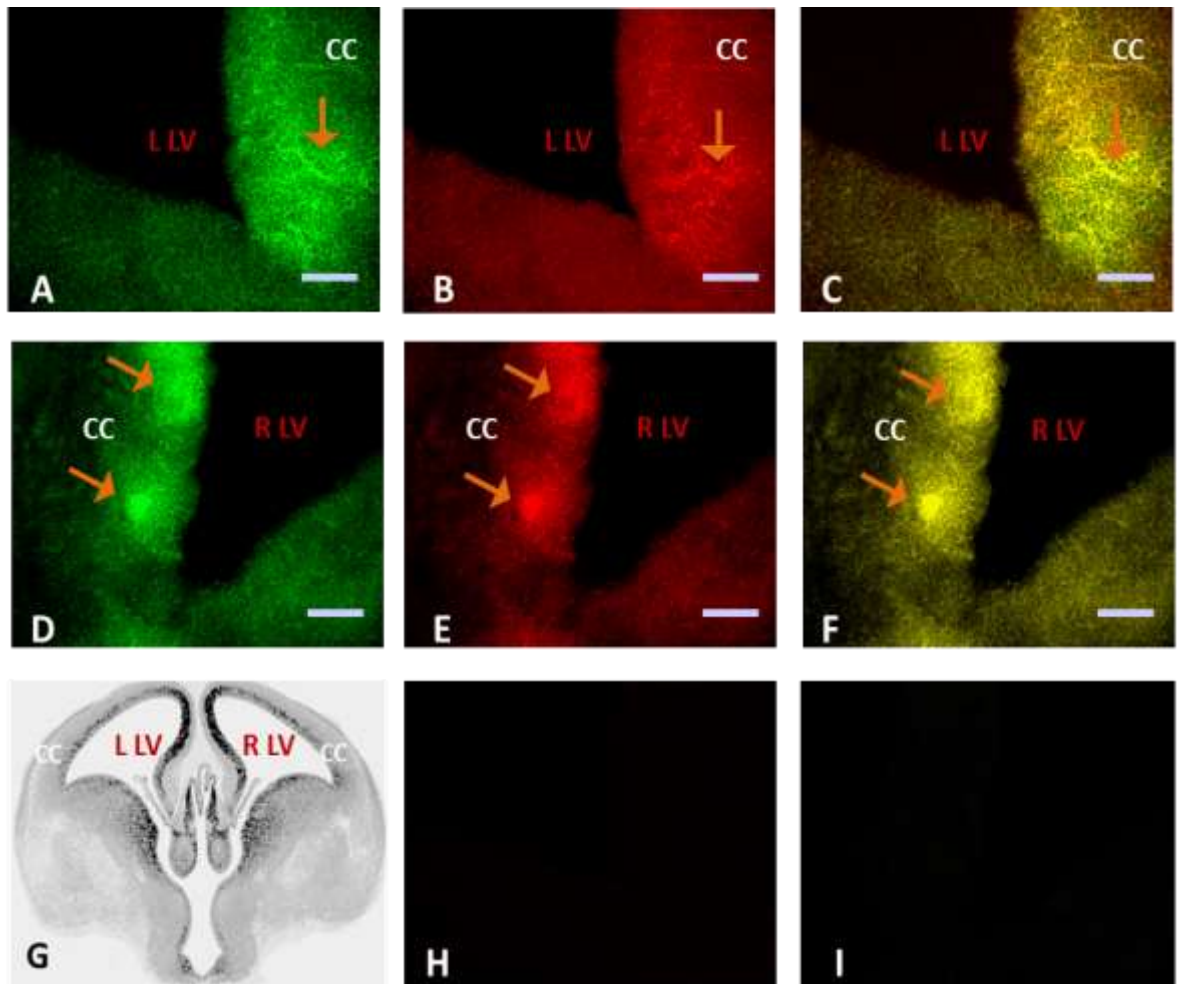


**Figure 2.15. Fluorescence photomicrographs of coronal sections (15 μm) show immunolocalization and colocalization of FDH and 5mTHF in cerebral cortex (CC) of right (R) and left (L) lateral ventricle (LV) at gestational age 18. Positive staining for FDH (green) was found in CC of normal brain (A) and hydrocephalic brain (D). 5mTHF (red) was also identified in CC of normal (B) and abnormal brain (E). FDH and 5mTHF co-localization was evidenced by the generation of yellow color after merging green and red channels (yellow), as appears in images (C) and (F). Illustrative scheme of brain structures with positive staining (G), Normal brain negative controls merged (H), abnormal brain negative controls merged (I) (Scale bar = 100 μm), (x40 magnification), N=6.**
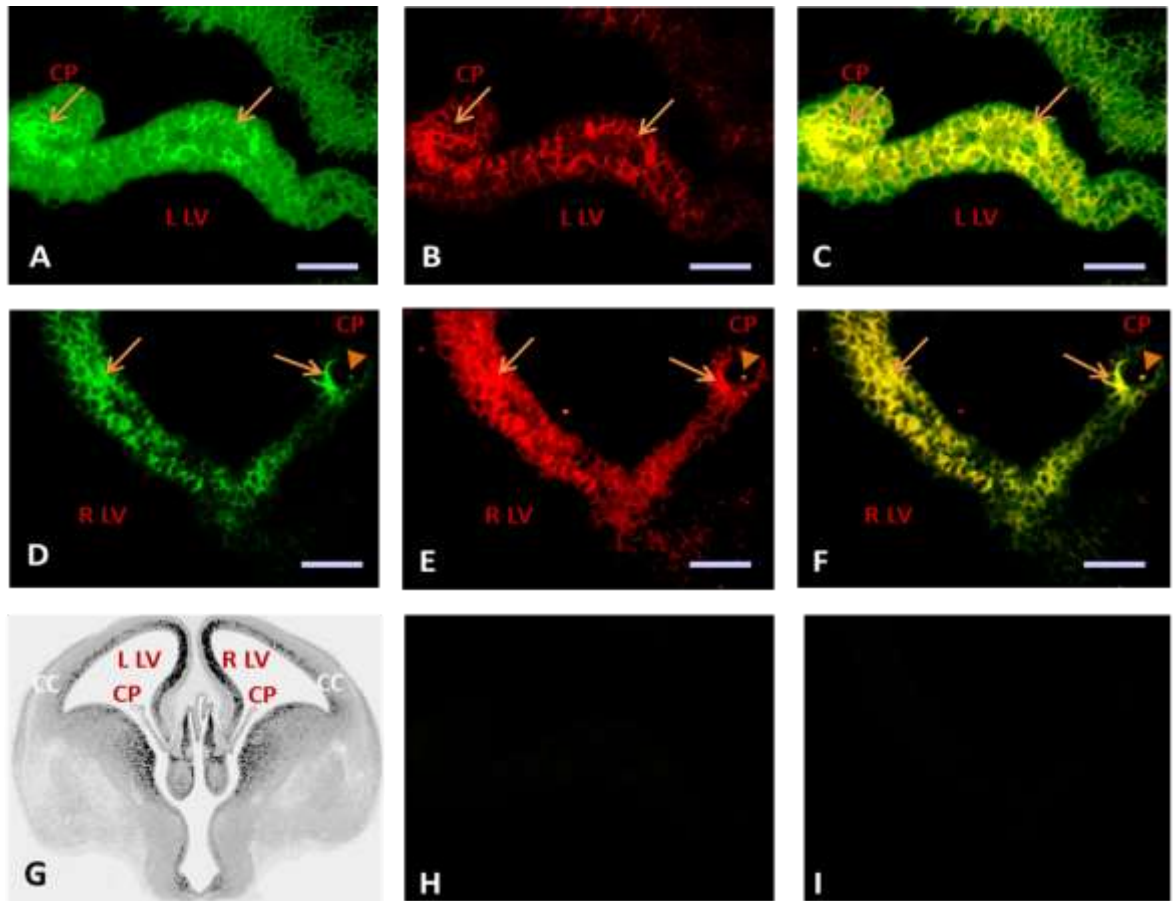
**Figure 2.16. Immunofluorescence staining (arrows) of coronal sections (15 µm) identified FDH (green) in CP of normal (A) and abnormal brain (D) in left (L) and right (R) lateral ventricle (LV). Immunoreactivity was also detected for 5mTHF (red) in CP of normal (B) and hydrocephalic brain (E) at gestational age 18. FDH and 5mTHF co-localisation was visualized by green and red channels merging of normal (C) and abnormal (F) brain images (yellow color). Vesicles-like structures (arrowheads) were observed in (D and E) and colocalized as shown in (F). Illustrative scheme of brain structures representing immunoreactivity in images is shown in (G), Normal brain negative controls merged (H), abnormal brain negative control merged (I) (Scale bar = 100 µm), (x40 magnification), N=6.**

Figure 2.15 shows heterogeneous FDH staining located on normal CC (A) and abnormal CC (D). A similar distribution is detected for 5mTHF in normal (B) and hydrocephalic brain (E). Surprisingly, and to our knowledge this was the first time it had been detected, choroid plexus showed FDH staining in normal brain (Figure 2.16 (B) and abnormal brain (E). Co-localization between FDH and 5mTHF was also detected, and is shown in figure 2.15 and 2.16 images (C and F) in both CC, and CP of normal and abnormal brain

respectively. The specificity of all antibodies was confirmed by a lack of detectable stain in negative controls.
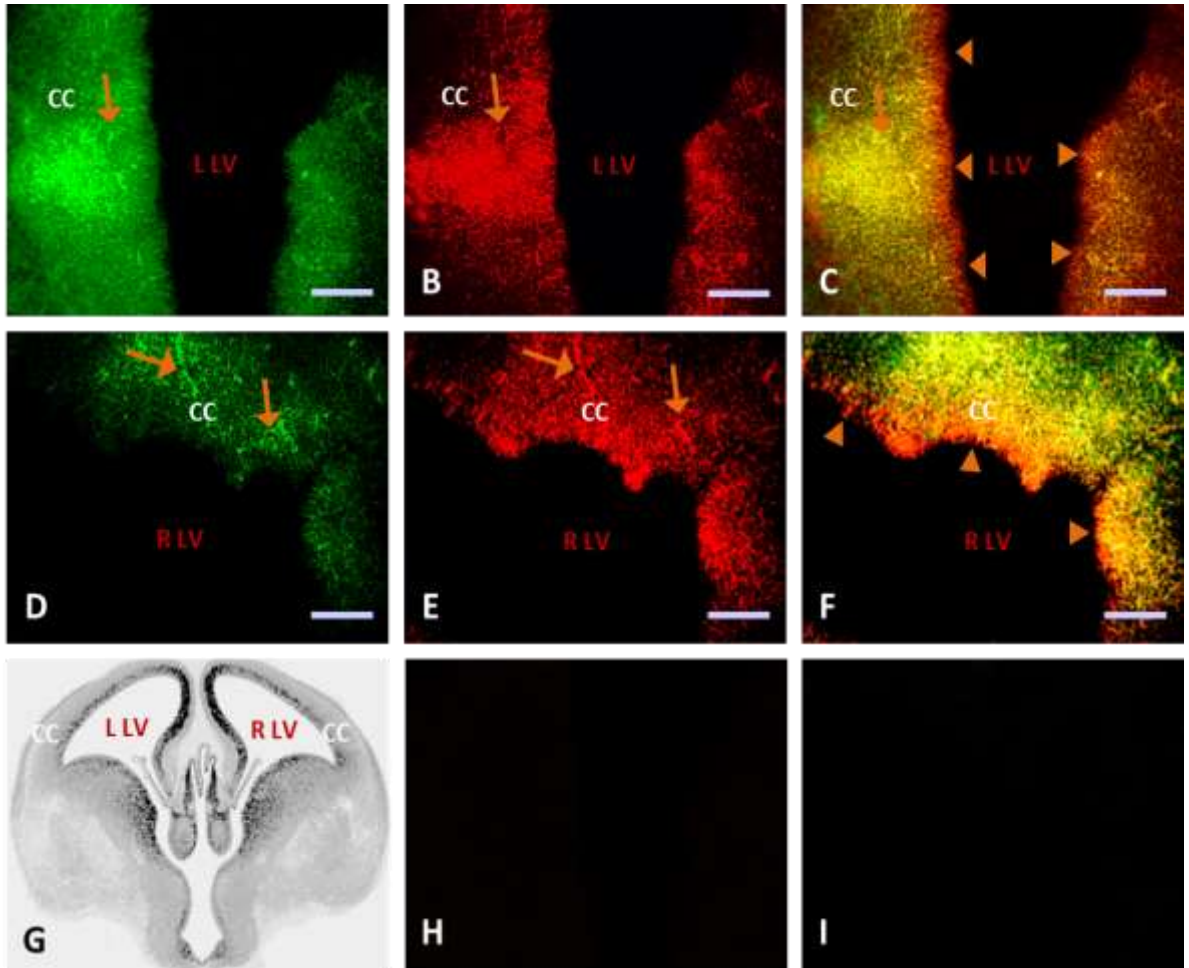


**Figure 2.17. Immunohistochemical staining of coronal sections (15 μm) identified FDH (green) in CC of normal (A) and abnormal brain (D) in left (L) and right (R) lateral ventricle (LV). Immunostaining was also positive for FRα (red) in CP of normal (B) and hydrocephalic brain (E) at gestational age 18. FDH and FRα co-localisation was exhibited by green and red channels merging of normal (C) and abnormal (F) brain images (yellow color). Illustrative scheme of brain structures showing immunoreactivity in images (G), Normal brain negative controls merged (H), abnormal brain negative control merged (I) (Scale bar = 100 μm), (x40 magnification), N=6.**
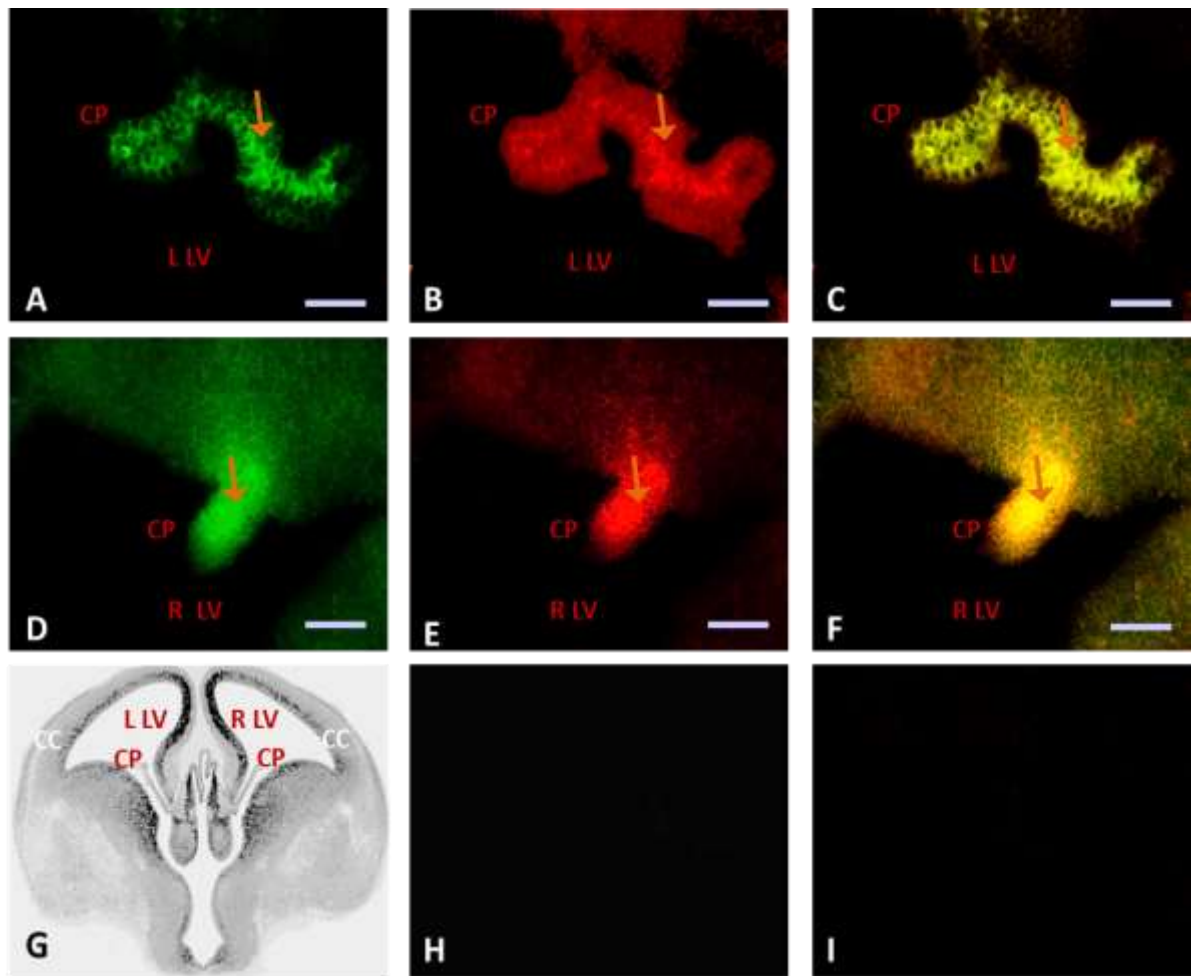
**Figure 2.18. Immunohistochemical staining of coronal sections (15 µm) show discernible staining for FDH (green) in CP of normal (A) and abnormal brain (D) in left (L) and right (R) lateral ventricle (LV). Immunostaining appeared also positive for FRα (red) in CP of normal (B) and hydrocephalic brain (E) at gestational age 18. FDH and FRα co-localisation was shown by green and red channels merging of normal (C) and abnormal (F) brain images (yellow color). Illustrative scheme of brain structures showing immunoreactivity in images (G), Normal brain negative controls merged (H), abnormal brain negative control merged (I) (Scale bar = 100 µm), (x40 magnification).**

In figure 2.17, discernible FDH and FRα staining was concentrated in specific areas, indicated with orange arrows on image A (normal brain) and D (abnormal brain) for FDH, as well as in images B (normal brain) and E (hydrocephalic brain) for 5mTHF. Figure 2.17 also clearly shows how FDH and FRα co-localize in CC in normal (C) and hydrocephalic brain (F). Interestingly, this pattern of distribution was similar to FDH and 5mTHF staining (Figure 2.15 and 2.16). Of notice, FRα staining was not only limited to staining in

the form of clusters co-localizing with FDH but also diffusing along the neuroepithelia (arrow heads). This is shown in Figure 2.17 in normal brain (C) and hydrocephalic brain (F). The pattern of distribution for FDH and FRα in CP is illustrated in figure 2.18, where FDH and FRα staining is exhibited again in the form of clusters (orange arrows) in normal brain (A; FDH) and hydrocephalic brain (B; FRα). A similar distribution is present in Texas rats' brain (D; FDH) and (E; FRα). In relation to protein co-localization, FDH and FRα co-localize in C (normal brain) and F (abnormal brain). The specificity of all antibodies was confirmed by lack of detectable stain in negative controls as shown in Figure 2.17 (H; FDH and I; FRα), and Figure 2.18 (H; FDH and I; FRα).
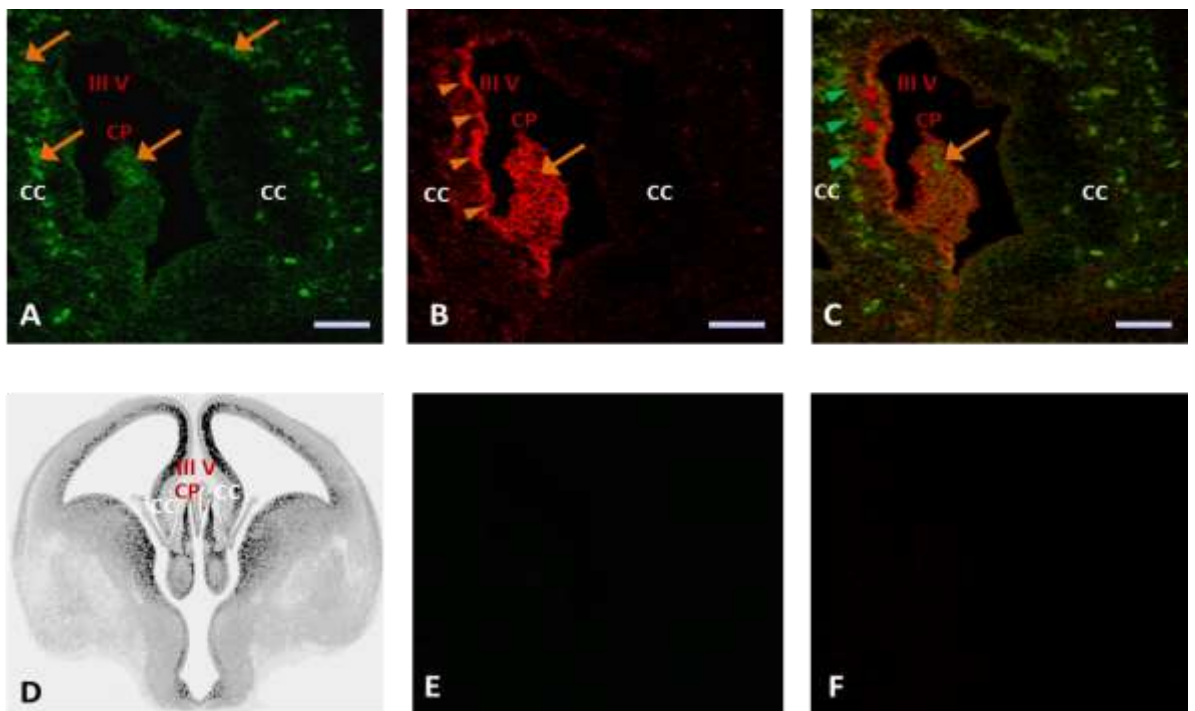


**Figure 2.19. Confocal images of fluorescent immunohistochemical staining show coronal sections (15 μm) of III normal brain ventricle (IIIV). FDH (green) is present in CC and CP (A, orange arrows) whereas FRα immunoreactivity (red) is exhibited in CC (B, red arrow heads) and CP (B, orange arrows). FDH and FRα proximity was shown by green and red channels merging (C; yellow color); see also arrow heads in green (FDH) and arrow heads in red (FRα). Illustrative scheme of brain structures showing immunoreactivity in images (D); normal brain negative controls (E); abnormal brain negative control (F); (Scale bar = 100 μm); (x40 magnification).**

Similar to results obtained from fluorescent microscopy images, confocal microscopy showed immunoreactivity for FDH in CC (A) and FRα in CP (B) of normal brain (Figure

142

2.19). However, this time the staining in CC (A) is punctuated and more dispersed, in contrast to the concentrated staining visualized on CP, which agrees with the FDH staining in aggregates detected  in fluorescent micrographs (Figure 2.16 and 2.18). Noticeably, FRα on the contrary is identified marginally along the neuroepithelia of the CC (B, arrow heads) but located in the form of clusters in CP (B, arrow), which matches the results previously shown for FRα immunoreactivity in CP in  fluorescent micrographs of normal and abnormal brain (Figure 2.15 and Figure 2.17). Importantly, after channels merge (C) FDH and FRα rather than co-localizing, they locate in close proximity, confirming confocal microscopy technology as a much more powerful tool for protein immunolocalization in fetal brain tissue. The specificity of all antibodies was revealed by the lack of detectable stain in negative controls (E; FDH) and (F; FRα). Overall, FDH, 5mTHF and FRα staining between CC and CP from normal and abnormal brains was of different intensity, and measurements of fluorescence density variability between normal and hydrocephalic brain were determined and plotted in Figure 2.20.
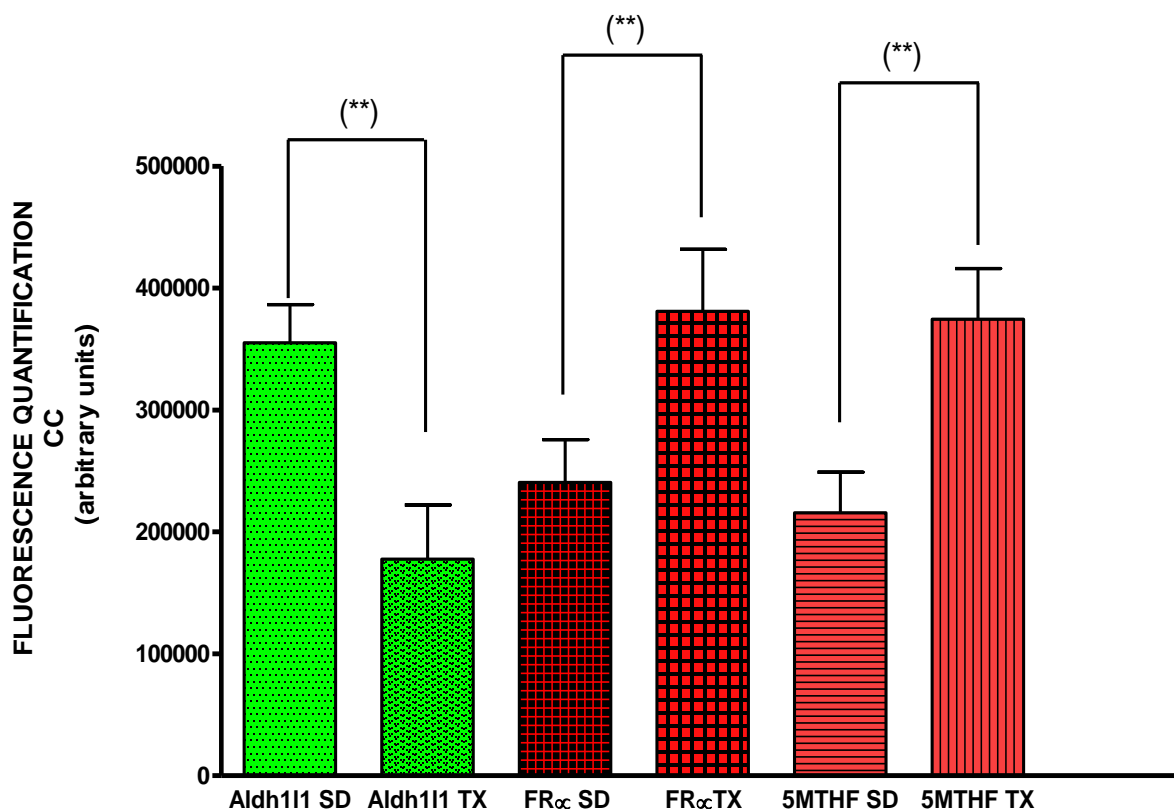
**Figure 2.20. Cerebral cortex (CC) immunohistochemical fluorescence quantification in Sprague Dawley controls (SD) and hydrocephalic rats. Fluorescence signal determination for FDH/ALDH1L1 (green), FRα (red squares) and M5THF (red lines) was assessed, as described in material and methods. The density of immunohistochemical staining was done per unit of area of section measured for CC in each group. Data from a minimum of 6 sections from each brain were averaged and recorded as mean ±SEM for all embryos in each category, calculated from all litters. Analysis of variance revealed significant differences between the cerebral cortex of SD and Texas rats for FDH, FRα and M5HTF at p≤0.01**

Fluorescence density measurements found in brain for FDH, FRα and 5mTHF were measured for CC and CP individually. Data from CC signal was pooled together and averaged. The mean values were compared across all SD and TX embryos as shown in figure 2.20. The differences between controls and hydrocephalic rats were determined using a two way-ANOVA analysis of variance. In general, FDH, FRα and 5mTHF staining presented differences in fluorescence density, when compared to staining between normal and abnormal brain. The comparative analysis revealed a mean fluorescence

density value for FDH staining significantly higher (281870±14403) in SD sections (green dot pattern) than hydrocephalic rats (177630±44518), (green and triangle pattern) p≤0.01. In relation to FRα staining, and in contrast to FDH results, SD control rats presented a significantly lower mean fluorescence signal (240602±35195), (red small square pattern) than TX rats (367626±65679), (red large square pattern). Similar to FRα, the 5mTHF staining average signal was less detectable in control rats than in hydrocephalic rats, with mean values of 194988±38899 and 321013±95042 in control brain and hydrocephalic brain respectively.

CP analysis was carried out following CC fluorescence measurements, using the same method to determine CC fluorescence signal. Detected density values were brought together and averaged resulting in a variety of differences, as shown in figure 2. 21below.
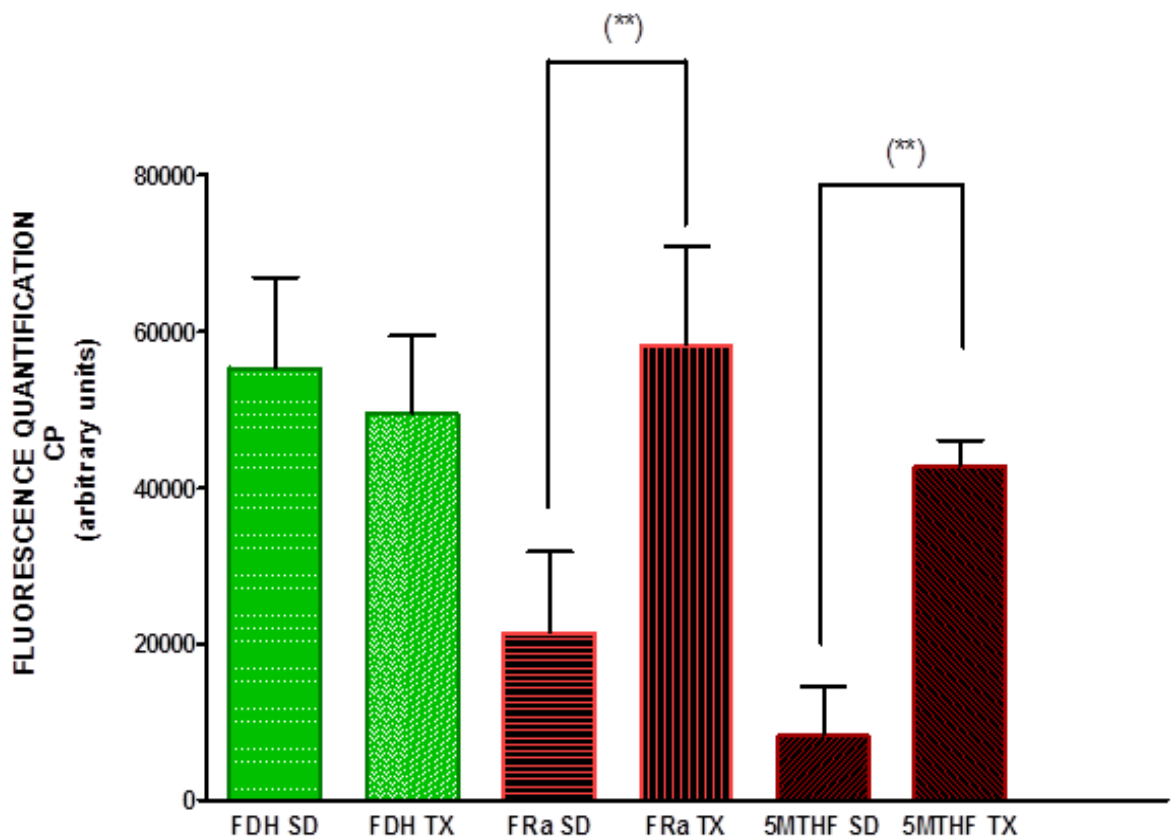
**Figure 2.21. Choroid plexus (CP) immunohistochemical fluorescence quantification in Sprague Dawley controls (SD) and hydrocephalic rats. Fluorescence signal measurement for FDH (green), FRα (red squares) and M5THF (red lines) was analysed as described in material and methods. The density of immunohistochemical staining was calculated per unit of area of section measured for CC in each group. Data from a minimum of 6 sections from each brain were averaged and presented as mean ±SEM for all embryos in each category (6), calculated from all litters. Analysis of variance revealed significant differences between the cerebral cortex of SD and Texas rats for FRα and M5HTF at $p \leq 0.01$**

Results from CP signal analysis in figure 2.21 show a minor decrease in FDH mean fluorescence when compared to normal (55326±11566) and abnormal brain (50266±9807). However, there was no statistically significant difference after analysis of variance ($p \geq 0.01$). With regard to FRα staining, control rats showed a much lower mean density value (20605±10290) than hydrocephalic rats (58438±11893). Finally, 5mTHF staining in

control rats exhibited a remarkable decreased signal (8326 ±6244) in comparison with abnormal rats (42717±3389). Immunohistochemical staining revealed too fluorescence signal in small spherical structures, which were occasionally noticeable in histological sections of CP (previous Figure 2.16) and CC (Figure 2.22). An intense positive FDH staining (A, green arrows) and FRα signal (red arrows) were detected inside these structures, which interestingly also co-localize (C, yellow arrows).

Since FRα is a cell membrane marker itself, we can only suggest that these spheres are vesicles containing FDH and FRα-5mTHF complexes. Finally, green arrow heads indicate FDH staining in CC and neuroepithelia of the striatum (NPCT), (A). Red arrowheads represent FRα staining in CC and NPCT, (B) and yellow arrowheads FDH and FRα co-localization.

In conclusion, results from fluorescence immunostaining confirm location and extensive co-localization of ALDH1L, FRα and 5mTHF in cerebral cortex and choroid plexus, as well as differential density of signal when contrasting normal and abnormal brain. Of notice, FDH location in CP was unexpected, and this finding does not agree with previous immunostaining results in our lab, where FDH passed undetected in CP. However, FRα results agree with previous publications that localize FRα in CP, CC and vesicles (exosomes). Our results clearly demonstrate for the first time co-localization of FDH and FRα along with 5mTHF, which give us more insight into the FDH's role in brain folate handling and interrelationships with folate transporters such as FRα.
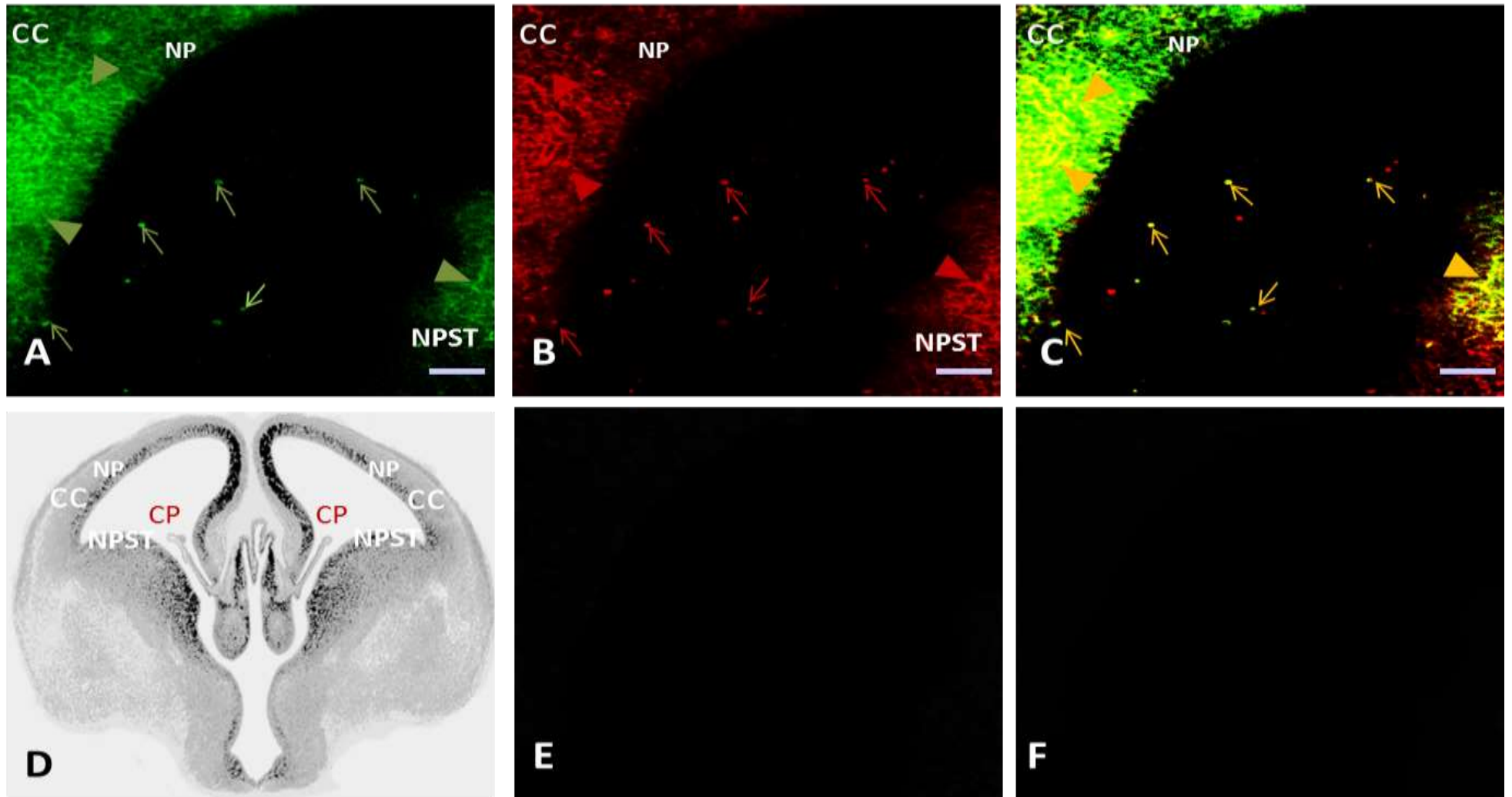
**Figure 2. 22. Representative fluorescence micrographs of FDH and FRα positive staining in CC, neuroepithelia of the striatum (NPST) and vesicle-like structures in LV. Green arrowheads show FDH staining in CC and NPST and green arrows indicates FDH in vesicle-like structures (VLS) (A). Red arrowheads point to FRα signal in CC/NPST and red arrows in VLS (B). FDH and FRα co-localization in CC and NPCT is shown as yellow staining (arrowheads) as well as co-localization of FDH and FRα inside vesicles which is indicated with yellow arrows (C). Scale bar 100μm.**

2.3.9 **FDH is significantly overexpressed in CC of the hydrocephalic brain.**

A relative quantification of FDH mRNA transcripts was carried out using traditional RTPCR technique, as described in material and methods, in normal and abnormal CC. This was done in order to elucidate, first, if there is a variation in FDH gene expression between normal and hydrocephalic CC, second, to assess whether FDH protein and mRNA coincide in location within the cerebral cortex, meaning CC synthesize FDH, and third, to determine whether changes in FDH protein levels correlate with changes in mRNA levels.
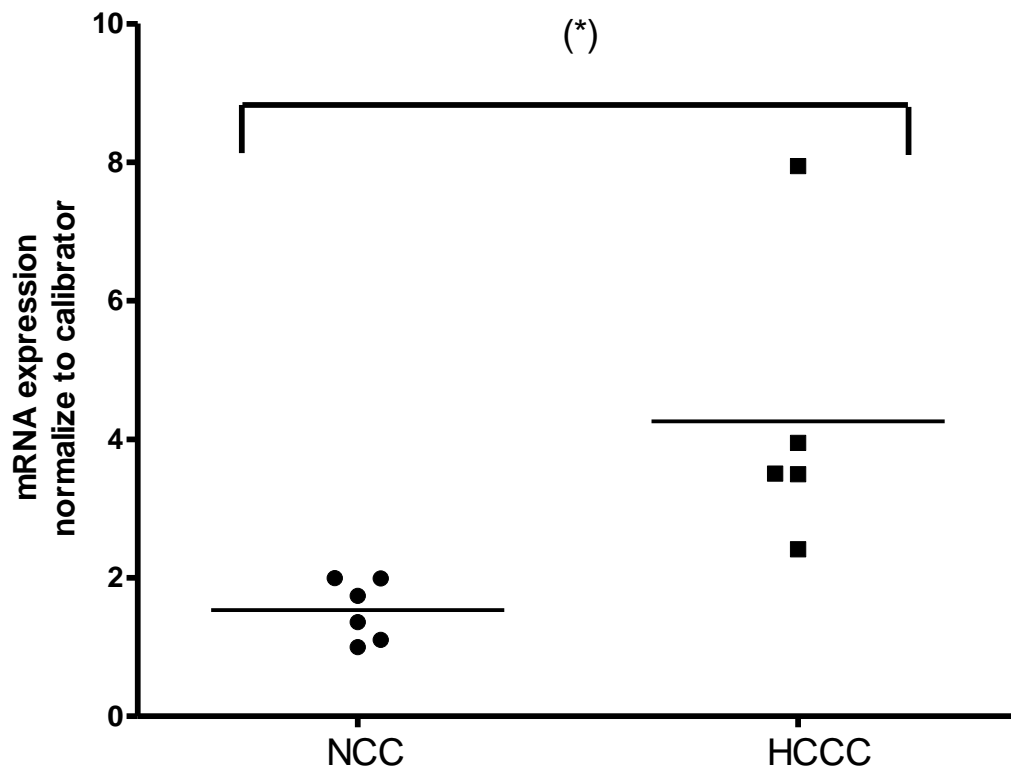


**Figure 2.23. Expression of FDH mRNA in normal cerebral cortex (NCC), indicated as circles; n=6 and hydrocephalic cerebral cortex cells (HCCC) represented with squares; n=5). mRNA expression was normalized to calibrator. Data are shown as a scatter plot with the median represented by the horizontal line *p < 0.05 (Mann-Whitney test).**

mRNA analysis for FDH and YWHAZ (reference gene) in normal and abnormal cerebral cortex cells, as well as liver (internal calibrator an positive control), was carried out in

parallel. All samples were amplified for b-Actin (housekeeping gene) to ensure cDNA integrity was not compromised.

Expression of mRNA for FDH was detected in all normal and hydrocephalic cerebral cortex cells examined with comparable expression between them (Figure 2.23). ALDHL1 expression in hydrocephalic cells was significantly higher than in normal cells *p<0.05, with Ct values in the range 29-30 and 27-28 for normal and hydrocephalic samples respectively. Ct value for liver was 27. A relative quantification using the comparative method was used to calculate the fold changes represented in graph (Figure.23) using the formula $2^{-\Delta Ct}$. Fold change is directly proportional to mRNA expression and hence the higher values in fold change were displayed in hydrocephalic samples also exhibiting the most variable pattern of expression between individual samples with fold changes in the range (2-8). By contrast, fold changes for normal samples were lower than for abnormal samples, with fold change values in the range (1.5-2). This outcome was unexpected as it was thought FDH protein synthesis should positively correlate with FDH mRNA transcription. However and contrary to our expectations, FDH was significantly overexpressed in fetal rat hydrocephalic brain.

Due to the particularly small size of fetal CP structures, it was impossible to obtain enough concentration of CP tissue to study ALDHL1 gene expression using the traditional RTPCR technique, and for this reason FDH mRNA in CP was assessed using ''in situ'' PCR instead.

## 2.3.10 **FDH mRNA is expressed in CC and CP in normal and hydrocephalic brain**.

According to our results from RTPCR, where ALDH1L mRNA were identified in cerebral cortex tissue, we proposed that FDH mRNA and protein location may also coincide in other regions of the brain, such as CP and vesicles. To ascertain if this was the case, and even more importantly, to verify if cerebral cortex relies on vesicles to acquire FDH and 5mTHF, a fluorescence technique suitable to identify mRNA rather than protein was chosen. This technique, called "in situ" RTPCR, was performed following the protocol described in material and methods for "in situ" PCR, to identify FDH mRNA transcripts in the entire brain. This technique allowed us to corroborate further the location of FDH in CC of normal and abnormal fetal brain, as well as to visually confirm gene expression of FDH in CP. As explained earlier, technical difficulties made it impossible to perform traditional RTPCR on CP tissue. This demonstrates the convenience of "in situ" PCR rather than the RTPCR technique to study FDH mRNA expression and location in fetal brain.

Figure 2.24 below shows a diffused and punctuated pattern of FDH mRNA staining (red color/Rhodamine, white arrows). Red signal is observed in the differentiating neuroepithelium of normal CC (NPCC), (B) and normal CP (NPCC, C). Some minimal but still positive staining is also observed in neuroepithelium of striatum (NPST), (C). In relation to hydrocephalic brain, a noticeable staining even at low resolution (x10) is identified marginally at the level of the NPCC (E). Signal in abnormal NPCC is also discernible in selected regions (square) as shown in image F (X40). Hydrocephalic CP presents a dispersed positive staining (H, x20), which can be seen more in detail in magnified selected region (square) in image I (x40). Staining is also localized in the differentiating hippocampal region (H) as indicated in images H and I. Negative controls

for normal brain (D) and abnormal brain (G and J) lack either forward or reverse primers, and there is no signal.
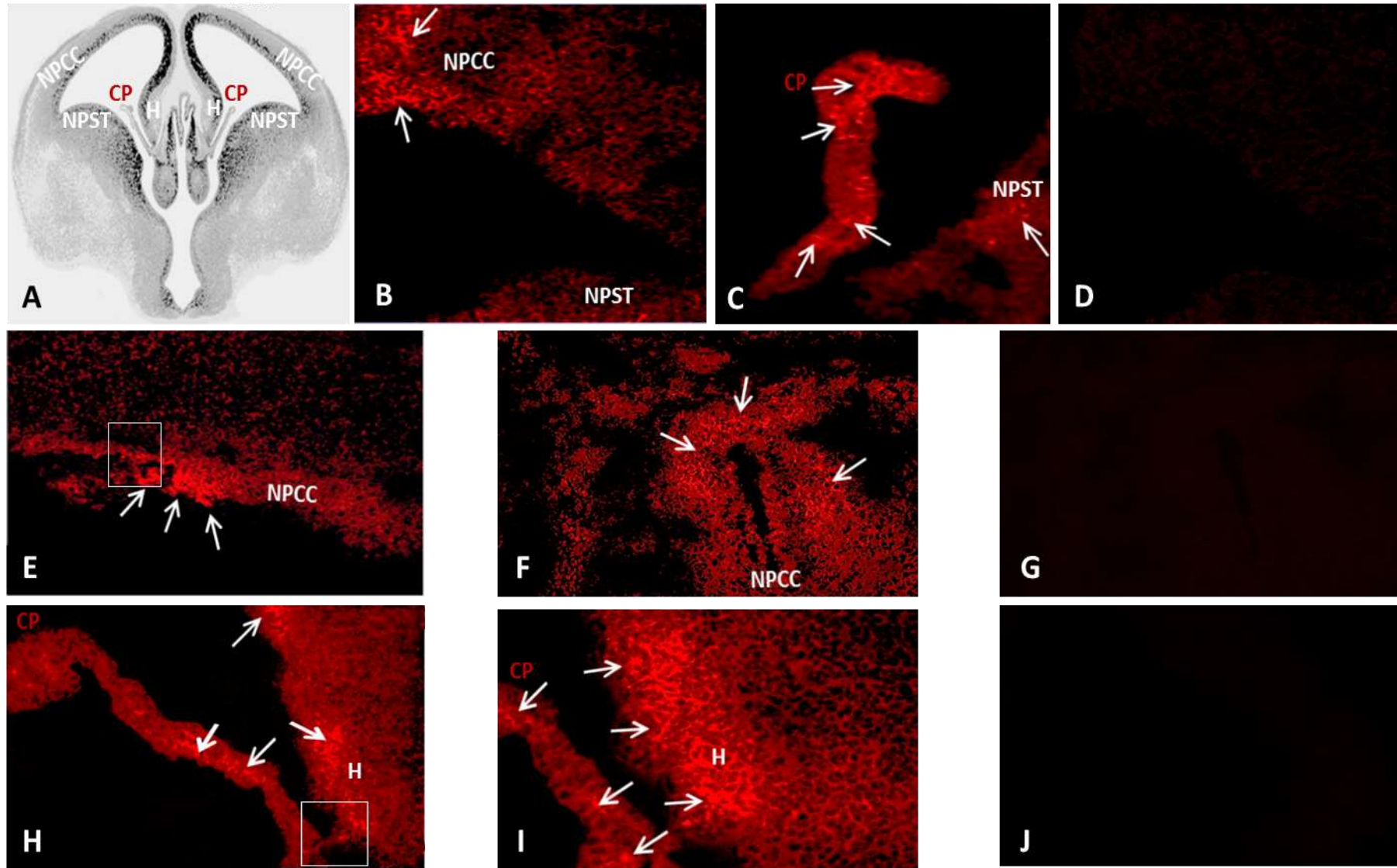
**Figure 2.24. Representative fluorescence (Rhodamine) micrographs of FDH mRNA tissue distribution in neuroepithelia of CC (NPCC) and CP (NPCP) in LV.** First illustration shows scheme describing brain structures shown in micrographs (A), positive labelling in NPCC and CP of normal brain (B) and (C) respectively (X40). Fluorescence signal was also detected in NPCC of hydrocephalic brain (E, x10) and selected magnified area (square) in F (x40) as well as in hydrocephalic CP (H, X20) and magnified area (square) in I, X40. Negative controls lacked forward or reverse primer as shown in (D) for normal brain, and G and J for abnormal fetal brain.

153

To demonstrate that the red signal in the images is due to PCR amplification of cDNA, rather than genomic DNA (gDNA), previously imaged brain sections (Figure 2.24) were subjected to DAPI staining and imaged again using confocal microscopy as described in material and methods. Blue nuclei can be observed in Figure 2.25 confirming the authenticity of the "In situ" PCR using primers designed uniquely to amplify FDH mRNA/cDNA, as also shown in figure 2.25. The FDH-mRNA/cDNA-Rhodamine labelled is exhibited in Figure 2.25 as intense red in B (normal brain) and C (hydrocephalic brain). On the other hand, the lack of red staining in nuclei revealed by blue DAPI staining confirms the suitability of the parameters chosen for the DNase treatment in our optimized protocol.

In conclusion, mRNA/cDNA location in normal and hydrocephalic brain demonstrates that fetal CC can synthesize its own FDH, and seems not to depend on vesicles coming from CP to obtain this enzyme. However, with this finding we do not exclude the possibility that fetal brain may also rely on FDH function as a folate binding protein and potential folate transporter to provide the CC with 5mTHF in the form of 5mTHF-FDH complexes, shuttled in vesicles coming from CP. This may take place in a similar fashion to FRα, a known folate transporter in charge of 5mTHF supply to the cerebral cortex through exosomes.
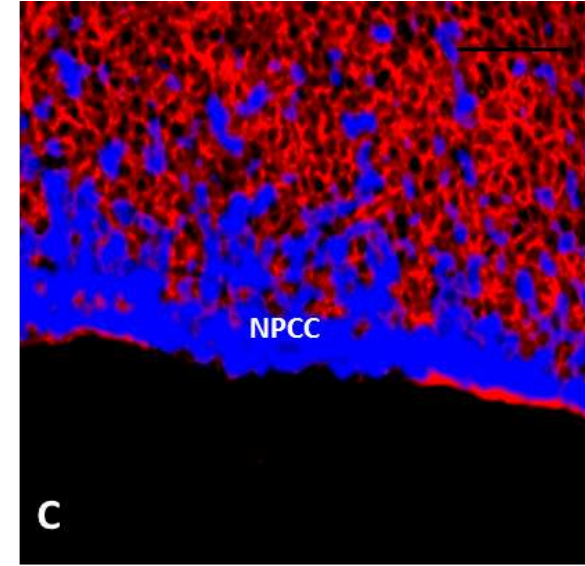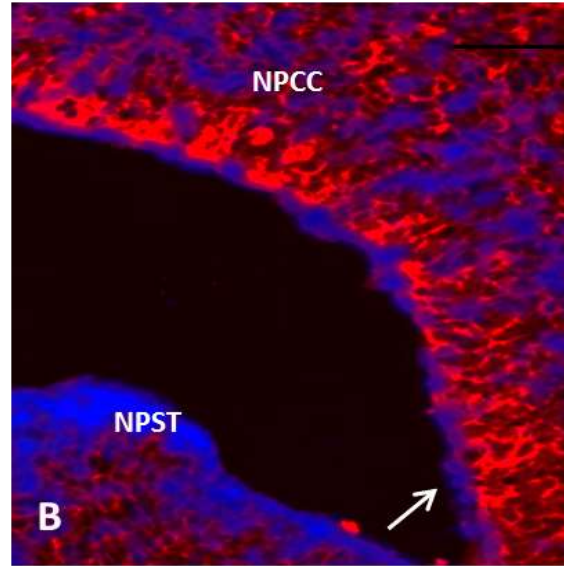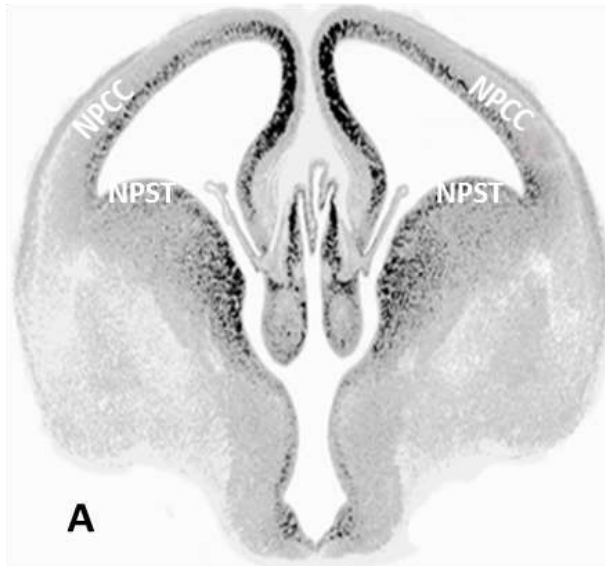
Normal brain. Image B clearly illustrates blue nuclei (DAPI staining) in NPST and NPCC. Blue nuclei is partially surrounded by Rhodamine staining (intense red) representing ALDH1L1 mRNA/cDNA in cytoplasm cells of NPST and NPCC. Vesicle-like formations are suggested in NPCC and NPST (white arrow). Scale bar (100 μ)

Abnormal brain. Image C shows intense blue stained (DAPI) in nuclei cells of NPCC. Rhodamine staining (red) reveals ALDH1L1 mRNA/cDNA production in cytoplasm cells of cerebral cortex. Scale bar (100 μ)

**Figure 2.25. Confocal images of double staining (DAPI+FDH mRNA/cDNA rhodamine labelled) on coronal brain sections (LV) treated with DNase (gDNA inactivation). Image A shows brain structures stained with DAPI (blue) and rhodamine (red) in normal brain (B) and abnormal brain (C). Positive control is shown below.**
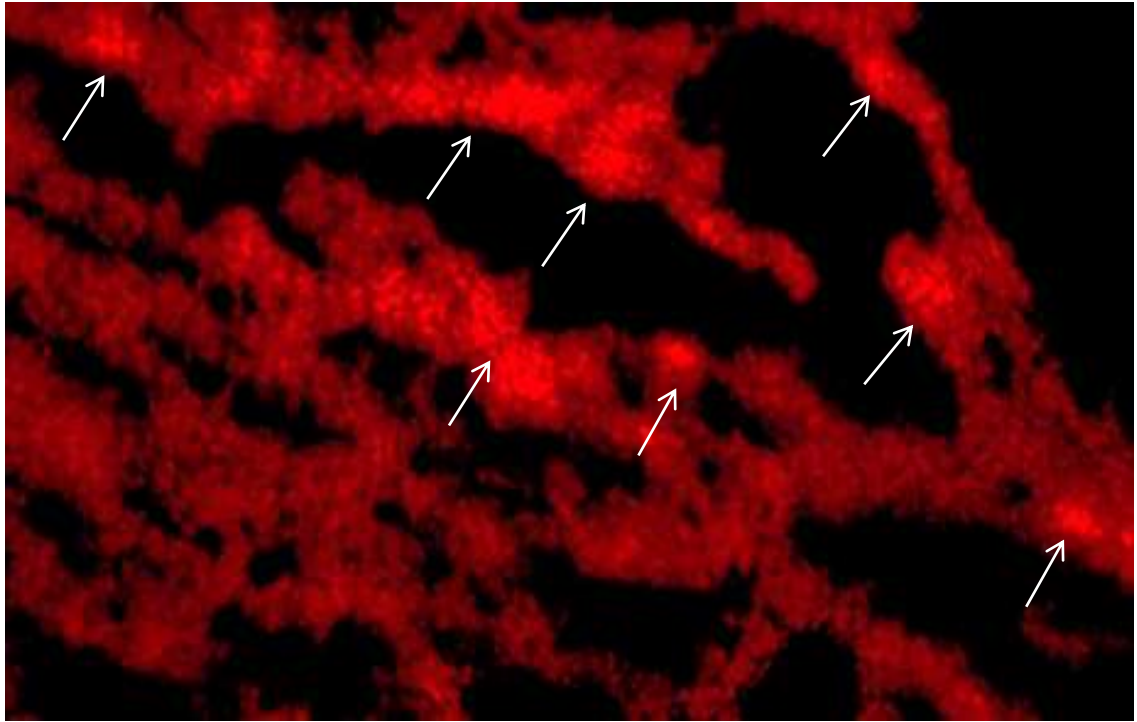
**Figure 2. 26. Fluorescence micrograph of liver, positive control for FDH mRNA/cDNA-rhodamine labelled amplification by "In situ" PCR. Image shows positive staining in hepatocytes indicated with white arrows (intense red), x40**

## 2.4. CONCLUSIONS-DISCUSSION

Previous research in our laboratory utilizing a combination of liquid chromatography and mass spectrophotometry (LC/MS) for rat CSF analysis identified differences in protein composition between normal and hydrocephalic CSF (Nabiuni, M. 2006). More than one thousand proteins were not present in hydrocephalic CSF when compared with normal CSF. One of the proteins missing in the abnormal CSF was the multicomplex and multifunctional folate enzyme FDH. This enzyme has been described in the past to have a role in human neural tube defects (Antony & Heintz, 2007) and it is associated with heritable folate deficiency in rodents (Champion et al. 1994). On the other hand, FDH is involved in the folate cycle, a major metabolic pathway dedicated to producing nucleotides among other important molecules in metabolism. Therefore, a lack of folate enzymes such as FDH in CSF also implies folate imbalance leading to nucleotide deficit followed by low DNA synthesis and diminished cerebral cortex growth (Nabiuni et al. 2006 and Cain et al. 2009).

In this study we investigated first the role of key folates in the presence and absence of FDH on cerebral cortex cells by means of "in vitro" cell culture experiments, cell bioavailability fluorescence quantification comparison between media conditions, and cell growth visualization by fluorescence DAPI staining. These experiments focused on 5mTHF, also called folic acid in its oxidized synthetic form (Powers, 2007), as 5mTHF is a key vitamin for cell growth "in vitro", as well as for the general development of the central nervous system in the first trimester of pregnancy (Scholl et al. 1996 and Darnton-Hill et al. 2015). As expected, results from cell cultures exposed to single 5mTHF showed a positive correlation between 5mTHF concentration and cell division. However, cell response to media containing 5mTHF and FDH was complex and strictly dependent on

concentration levels of each molecule. When the active form of the enzyme was added at normal rat CSF concentration levels ($10^{-5}$ M), and 5mTHF was present at high concentration in the range $10^{-4}$-$10^{-6}$ M, cells proliferated significantly (p<0.01). In contrast, active FDH promoted growth when 5mTHF was at low concentration ($10^{-7}$-$10^{-9}$ M), although this result was no statistically significant (p>0.05). In view of this outcome, it is proposed that active FDH binds to 5mTHF when present at high concentration, storing it for later use when needed. On the other hand, when 5mTHF is at low concentration, both 5mTHF and active FDH are free to enter the cells and activate the folate metabolic cycle, encouraging cell growth.

We also worked with an inactive form of the FDH (point deletion in intermediate domain) to study its folate binding properties in isolation and its effect on growth in the presence of 5mTHF. On this occasion, FDH at the same concentration as previous experiments ($10^{-5}$) was significantly detrimental for cell growth (p<0.01) when 5mTHF was present at nearly all concentrations in the range ($10^{-4}$-$10^{-9}$). These results were similar to results obtained with active FDH and 5mTHF at high concentration, which suggests that inactive FDH sequesters 5mTHF, acting as a 5mTHF extracellular reservoir, and impeding 5mTHF access into the cells, thus leading to a generalized decrease in cell growth.

Similar results were obtained from experiments with media containing constant 5mTHF ($10^{-8}$ M) and active FDH at high concentration (10-5 x $10^{-5}$), where a relatively significant percentage decrease (12%) (*p<0.005) in cell growth occurred. Yet, this decrease in cell proliferation was less remarkable under these conditions than the reduction in cell growth seen with experiments using constant active FDH and high 5mTHF concentration, where the percentage decrease in cell proliferation was greater than 40% when compared to the

media control.  Finally, an overall reduction in cell growth was also observed with inactive FDH at all concentrations in the presence of constant 5mTHF p<0.005.

In conclusion, "in vitro" experiments for the study of 5mTHF's effect along with FDH on cell proliferation suggested that FDH has affinity for 5mTHF, and this affinity depends on the specific FDH and 5mTHF concentration in the extracellular media.

In view of previous results, a sandwich ELISA was developed to investigate further whether or not there is affinity of FDH for 5mTHF. This approach aimed to clarify the suspected linkage between the enzyme and the folate 5mTHF in "in vitro" cell culture experiments. Sandwich ELISA immunoassays have proven a valuable asset for the detection of analytes of interest in body fluids.  To investigate the potential affinity of FDH for 5mTHF further, an "in house" fluorescent immunoassay was developed. The immunoassay was able to detect FDH-5mTHF binding in all serial dilutions, producing a decrease in signal directly proportional to FDH and 5mTHF concentration. The intra and inter variation coefficient results were also relatively high, at 9.2 and 9.55 respectively, confirming the need for further optimization of the assay. For instance, polyglutamated 5mTHF may be used instead of the non-glutamated 5mTHF used in this assay as it is known FDH is affected by the degree of folate polyglutamation (Kim, D.W. et al. 1996 and Anguera M.C. et al 2006). In spite of the need for assay refinement, the Sandwich ELISA was adequate to confirm FDH affinity for 5mTHF. This outcome suggests a new role for ALDHL1 as a 5mTHF binding protein, similar to FRα, a membrane receptor with a high affinity for reduced folates such as 5mTHF (Steinfeld, R. et al. 2009, Cains, S et al. 2009 and Zhao et al. 2011).

Furthermore, the confirmation of FDH affinity for 5mTHF suggests the potential ability for this enzyme to pool free extracellular and intracellular 5mTHF, and agrees with previous publications about a possible function for FDH in the regulation of folate storage (Anguera et al. 2006). In this respect, a lack of FDH in CSF can be detrimental for regulation of 5mTHF concentration, having a negative knock-on effect on different areas of the brain. Importantly, this outcome also indicates that FDH may have a role in methylation, providing methyl groups for DNA modifications and SAM production, SAM being a methyl donor which is involved in more than 100 metabolic reactions. Interestingly, it is known that altered homocysteine and methionine levels are associated with the narrowing of blood vessels, such as the carotid arteries in the brain and blood vessels in the placenta, leading to abruption and pre-eclampsia (Solansky et al. 2010). Also, recently, high homocysteine levels in plasma have been linked to blood vessel global hypomethylation (Yang et al. 2014). Because methionine production is dependent on methyl group provision to the folate metabolic cycle through 5mTHF, we suggest that the low FDH levels observed in hydrocephalic CSF also mean that 5mTHF deficit and perturbed balance between methionine and homocysteine plasma levels put pregnancies and fetal development at risk.

In addition, 5mTHF is a substrate for the ubiquitously produced methionine synthase (MTR) (www.genecards.com), which is responsible for the conversion of 5mTHF into THF. THF is a folate at the core of the folate metabolic cycle, needed among other functions for nucleotide production and concomitant production of methionine (Refsum et al. 2001) an amino acid which is vital for fetal brain development (Davis et al. 2004 and Dauncey, MJ et al. 2012). Nevertheless, THF is also FDH's product of reaction (Schirck et al. 1994 and Kim et al. 1996), for which FDH has an affinity greater than for its substrate

(Schirch et al. 1994). For this reason, THF's effect on cell proliferation was also analysed in the presence and absence of FDH. Results from these experiments were contradictory; whilst THF with and without FDH in the media did not have any significant impact on cell division, experiments using THF conjugated to a THF fluorescent IgG led to an increase in cell proliferation. The differences in cell growth impact by single THF compared to THF linked to immunoglobulins were due to the unstable nature of this vitamin. Single THF reacts with oxygen especially in neutral or alkaline solutions at room temperature, rendering it inactive because of degradation after oxygen binding and loss of protons, causing a change in THF chemical properties and structure. In this regard, we minimized THF degradation by purging THF solutions with Argon, a gas which is able to remove oxygen and therefore prevent inactivation. However, THF's contact with oxygen was unavoidable once added to the media which was included in cell cultured plates, which were in contact with air at room temperature. Due to cell culture media air exposure in the incubator, THF must have degraded after 24 hours of growth, as it is known to become 10% inactive after 5 minutes at 37°C and 25% inactive after 45 minutes in the presence of oxygen (Young, D. W. et al. 1969). Interestingly, fluorescent IgG conjugated to THF potentiated cell growth in comparison with negative control media, as seen in fluorescent IgG-THF tracking experiments. These results strongly support the suitability of immuno-stabilized THF for prevention of oxidation, encouraging cerebral cortex cell growth "in vitro", which was confirmed by fluorescence quantification assay and DAPI staining of primary cell cultures containing the IgG-THF complex. Nevertheless and in spite of acknowledging that DAPI staining does not allow assessment of cell bioavailability, we argue that in primary cell cultures the majority of cells are alive and therefore fluorescence quantification assay of cell growth and the DAPI staining exhibited by the cells nuclei represents cell bioavailability (Davis, J. 2009).

In conclusion, the "in house" fluorescent IgG-THF conjugate and its positive effect on cell growth foresee a potential folate treatment for congenital hydrocephalus and prevention of the cortical plate gross reduction described in fetal brain during this condition. This outcome is also in accordance with previously published research in our lab, where THF in combination with folinic was also beneficial to increase cerebral cortex growth "in vivo" (Cain et al. 2009). Because folates are thought to be transported bound to their enzymes or their receptors in plasma and CSF (Jones et al. 2002), it is not surprising that stabilized THF had a better impact on cell growth than unstable THF, demonstrating the inability for natural THF to be active freely in body fluids. In this scenario, these results also provide evidence that THF may be transported bound to FDH in CSF, a consideration, as mentioned in the introduction to this research, which is also plausible for 5mTHF. In any case, a more extensive investigation needs to be carried out to study the potential use of THF-IgG complex as a folate supplement for congenital hydrocephalus "in vivo".

With regard to folic acid, cell proliferation with media enriched with the artificial folate alone had the greatest positive effect on cell growth, with percentage increases higher than 80% when compared to the media control. The cell response was similar with folic acid in combination with FDH. Surprisingly, these results were opposite to results from previous research "in vivo" in our lab group, where folic acid supplementation to the hydrocephalic mother was unsuccessful to treat congenital hydrocephalus. This highlights that "in vitro" results do not always coincide with "in vivo" results. Nevertheless, "in vitro" experiments were chosen as a first step for the purposes of our studies.

To clarify whether FDH is either located and biosynthesized in cerebral cortex, or transported in vesicles from choroid plexus within exosomes in a similar fashion to FRα

transport system (Grapp et al. 2013), or both, fluorescence FDH protein and mRNA location, as well as gene expression studies, were carried out on fetal brain at embryonic day 18, when CSF blockage and accumulation is established in the hydrocephalic brain.

Since immunohistochemical studies localized ALDHL1 with 5mTHF and FRα in choroid plexus, as well as in cerebral cortex, we can only suggest that FDH and FRα are interrelated and share a common transport function, which is the handling and provision of 5mTHF to the different areas of the brain. With regard to the vesicles identified in some brain sections, these were detected thanks to FRα being a membrane marker itself. These vesicles too showed colocalization of FDH with 5mTHF and FRα in the LV in proximity to choroid plexus and neuroepithelia of areas in the process of differentiation. Previous work published by Bachy et al. in 2008 and Harrington et al. in 2009 supports these findings. In addition, it is known that differentiation is an on-going process at embryonic day 18, and for this reason it is not surprising that these vesicles appear close to the neuroepithelia of the cerebral cortex and the striatum, confirming a need for 5mTHF in both regions of the brain which are in the process of differentiation. Furthermore, these findings also provide evidence of a role for vesicles in the transport for FDH-5mTHF and FRα-5mTHF complexes to different areas of the brain, and concur with Grapp et al.'s research in 2013 and their discovery of vesicles (exosomes) with a role in transporting FRα-5mTHF complexes from choroid plexus to cerebral cortex.

Based on the assumption that ALDHL1 protein and mRNA should coincide in location, "in situ" RTPCR was performed to identify mRNA in normal and abnormal brain. A noticeable staining was exhibited by the choroid plexus and cerebral cortex in both normal

and abnormal brain. However, time restrictions to the duration of this project did not allow gene expression analysis and location of FRα mRNA. In any case, the Steinfeld research group in 2009 published their studies about mRNA expression of folate transporters in human brain tissue finding high levels of FRα mRNA expression in choroid plexus of the adult brain, but not in fetal brain. In view of their results and our findings, we propose that the cerebral cortex relies on FRα for the provision of 5mTHF. However, FRα is only responsible for 50% of the folate transport in the brain (Wollack et al. 2008) and in spite of other brain folate transporters such as Reduce Folate carrier (RFC) (Wang et al. 2001) and Proton coupled folate transporter (PCFT), known to be involved in transport (Ganapathy et al 2004, Wollack et al. 2008), only FRα and FRα-PCFT dependent transport has been proven as the main folate transports in the brain (Grapp et al. 2013). Taking into account these findings and our results about FDH affinity for 5mTHF and its co-localization with FRα and 5mTHF, we can only conclude that FDH is involved along with FRα in the transport of 5mTHF from choroid plexus to cerebral cortex through vesicles (exosomes) formed in the choroid plexus and floating in CSF (Bachy et al. 2008). On the other hand, FDH mRNA expression analysis by traditional RTPCR and "in situ" RTPCR confirmed that FDH is produced in choroid plexus and cerebral cortex, suggesting that cerebral cortex acquires FDH through vesicles, as well as being able to produce the enzyme "in situ". Going back to our "in vitro" experimental results using media containing FDH and 5mTHF, we proposed that FDH acts as a folate transporter binding to 5mTHF in situations of high 5mTHF concentration.  Therefore, FDH seems to act as a folate binding  protein to either transport 5mTHF or store it when present in excess at extracellular level in body fluids, such as CSF, as well as intracellularly.  This possibility was also considered by Krupenco's research group in 2010.

With regard to differences in fluorescence density analysis of FDH staining between normal and abnormal brain, it should be emphasized that FDH protein levels in abnormal brain were lower than in normal brain (p≤ 0.01), and this result did not correlate with mRNA expression in cerebral cortex. On the contrary, FDH mRNA expression was higher in hydrocephalic brain than normal brain (p≤0.05). A plausible explanation for this outcome is that gene expression and protein levels do not always correlate. This is due to differences between enzymes and mRNA stability; whereas protein half-life can be hours or days, mRNA degradation is limited to 2-7 hours. Furthermore, mRNA transcription rate and protein translation rate do not always correlate either, and normally only when the protein is highly abundant is mRNA also highly expressed. Finally, there are mRNA posttranscriptional modifications and protein posttranslational changes that contribute to variations in the total level of mRNA and protein leading to uncorrelated results (Vogel et al. 2012).

With regard to FDH immunohistochemical studies, it was found that there is a protein deficit at tissue level in the hydrocephalic brain, which is in agreement with results from previous research where hydrocephalic CSF was devoid of FDH. Even more importantly, results from FDH gene expression analysis showed high mRNA expression in abnormal cerebral cortex, which we argue may be a response to low FDH concentration in CSF. Interestingly, this is the case for FRα regulation of gene expression where biosynthesis of FRα is activated in situations of low extracellular folate by negative feedback (Anthony 1996). Negative feedback may be followed by problems at transcriptional or translational level, causing low synthesis of FDH intracellular and/or extracellularly.

With respect to FRα and 5mTHF, fluorescence density analysis of staining showed major protein expression in abnormal brain when compared to normal $p \leq 0.01$. This agrees with previous publications of our work where the Dot Blot technique showed that 5mTHF was significantly more abundant in hydrocephalic fetal CSF than in normal brain at embryonic day 20. Interestingly, the level of 5mTHF in the mothers' serum was significantly lower in Texas rats than in normal rats (Cain et al. 2009). Considering that FRα is not produced in the fetal brain (Steinfeld et al, 2009), we can only argue that FRα-5mTHF complexes come from blood circulation in contact with the highly irrigated choroid plexus (Dziegielewska et al 2001 and Hartwig et al. 2010), and that FRα gene expression control and protein production happen somewhere else upstream, rather than in the brain. For instance, regulation of FRα protein expression may be controlled by the placenta, an auxiliary organ known as an important source for FRα synthesis during pregnancy (Henderson et al. 1995, Elnakat & Ratnam 2001 and Solansky et al. 2010). From this point on, we argue that the placenta rather than fetal brain may be responsible for the differences in FRα, and hence, the 5mTHF differences in concentration seen between hydrocephalic and normal brain. We therefore propose the hydrocephalic placenta as an organ for future folate-transport investigation. The role of placenta in maternal-fetal vitamin transport in humans was established by Baker et al in 1981.

Furthermore, since 5mTHF is a ligand for FRα and, as suggested in this study, also a ligand for FDH, and taking into account the biding ratio should be 1:1, higher levels of 5mTHF were expected to be found in both normal and abnormal brain. Therefore, not all FRα and FDH molecules bound to 5mTHF, which suggests that competition led to a low rate of binding. This is commonly observed in receptor-bivalent ligand binding studies (Urbana, 1993 and Xie et al. 2005).

Finally, our findings reveal high concentration of FRα and low FDH protein in Texas rats at tissue level. Increased levels of FRα may lead to exacerbated 5mTHF provision from choroid plexus to cerebral cortex. At the same time, FDH is considered to be a regulatory protein with a role in creating folate pools in situations of high intracellular concentration, storing them for later use in the case of high demand (Anguera et al. 2006 and Krupenco et al. 2002 and 2010). Consequently, our findings (low FDH protein levels) mean free one-carbon folate molecules and folate excess in CSF, as well as intracellularly, leading to CSF folate imbalance and homeostasis disruption, all of which have negative consequences for CNS development as a whole. Accordingly, a role for folate receptors in homeostasis has been established in the past (Weitman et al. 1992 and Johansson et al. 2008).

In conclusion, there are three functional implications for congenital hydrocephalus arising from these studies. First, FDH affinity for 5mTHF outcomes suggests FDH is able to bind to 5mTHF. This finding means that at extracellular level, FDH may transport 5mTHF into vesicles, providing the cerebral cortex with this key folate. At intracellular level, FDH binding to 5mTHF may have a regulatory function in that it aims to store 5mTHF in situations of high 5mTHF concentration. Our findings also revealed low concentration of FDH at tissue levels, which implies low regulation of 5mTHF concentration and folate cell homeostasis disruption. This may also be the case in situations of low FDH in hydrocephalic CSF. Secondly, FDH mRNA overexpression by hydrocephalic cerebral cortex cells did not correlate with the levels of protein found. Based on these results, we conclude that FDH mRNA transcription and/or translation processes may also be altered in congenital hydrocephalus, resulting in low FDH protein expression. On the other hand, the low concentration of FDH in hydrocephalic CSF found in previous published studies, and the high mRNA expression in cerebral cortex seen in our studies, suggest that a negative

feedback loop is occurring to compensate for the low levels of FDH present extracellularly. Thirdly, we argue that the high levels of FRα and 5mTHF observed in choroid plexus and cerebral cortex come from the blood supply irrigating the choroid plexus, as FRα is not expressed in either choroid plexus or cerebral cortex. In this regard, we propose that the placenta is also impaired in hydrocephalus, resulting in FRα gene overexpression. Finally, our results in relation to stabilized THF's positive effect on cell proliferation suggest new avenues for the development of a safe treatment for congenital hydrocephalus, instead of the life-threatening invasive surgery (shunting) commonly used these days.

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# CHAPTER 3

# FOLATES ARE CRUCIAL FOR FETAL MENINGEAL ARACHNOID CELL GROWTH: IMPLICATIONS FOR CONGENITAL HYDROCEPHALUS.

## ABSTRACT

Past investigations revealed an association between reduction in the CSF concentration of the folate binding enzyme 10-formyltetrahydrofolate dehydrogenase (FDH), reduced cerebral cortex development and hydrocephalus. We propose that FDH and folates are also crucial for the development of a functional arachnoid, which is in charge of normal CSF drainage and brain CSF outflow. Impediment of normal folate transport caused by low FDH levels in CSF, and therefore low folate provision to the arachnoid tissue, may lead to arachnoid dysfunction and consequently CSF flow blockage and congenital hydrocephalus. The present study aims to investigate the role of FDH in folate transport and its contribution to the development of the arachnoid tissue in the fetal brain.

## 3.1 INTRODUCTION

The arachnoid is a secondary blood-cerebrospinal fluid barrier, and one of the three layers of connective tissue (meninges) enclosing the brain and the spinal cord. The arachnoid matter starts developing at embryonic day 13.5 and is made up of fibrous tissue linked by tight- junctioned epithelial cells (Bhavya et al. 2007). Contiguous to the arachnoid runs another meningeal layer, the Piamater, a meninge also made of arachnoid tissue with strands connecting with the arachnoid membrane and forming a three-dimensional web (Kida et al. 1988). The space between the arachnoid and the pia is known as the subarachnoid space and is filled with cerebrospinal fluid (Boulton et al. 1999). At gestational day 18, CSF synthesis commence in the primary blood-cerebrospinal brain barriers, the choroid plexuses, which are small structures situated inside the brain ventricles (Oi et al. 1996). CSF is constantly produced and flows from ventricles to the SAS, leaving the brain mainly through the arachnoid villi in the arachnoid (Xin et al. 2010). Despite over 100 years of research into the arachnoid tissue, its involvement in CSF flow and its role in drainage are still poorly understood (Glimcher et al. 2008 and Holman et al. 2010). A great deal of importance has been attributed to specific structures in the mature arachnoid (arachnoid granulations) in terms of their role in molecule transport and in allowing small molecules to be drained into blood, whilst preventing large molecules in CSF from unloading into the venous sinuses in contact with the arachnoid tissue (Grzybowski et al. 2006 and Glimcher et al. 2008). It is known that blockage of CSF absorption in the arachnoid can give rise to an altered flow and CSF accumulation, leading to congenital hydrocephalus. Thus, congenital hydrocephalus has been associated with arachnoid development impairment and dysfunction due to abnormal morphology and altered differentiation of this meningeal tissue (Vivatbutsiri et al. 2008). However, the specific pathology of congenital hydrocephalus remains unclear, and the condition has

been reported to be more than just an alteration in CSF flow and mechanical phenomena. Children born with hydrocephalus present with neuroepithelia denudation, leading to Sylvius Aqueduct obliteration and CSF obstruction, impairment of the cerebral cortex and cognitive deficit. These events are thought to be caused by abnormal development of certain cerebral neuron's populations and abnormalities of the subcomissural organ, which in turn results in changes in CSF composition, severe alterations of the hypothalamus and neuroendocrine deficiencies (Miyan et al. 2003 and Rodriguez et al. 2006).

CSF contains folates; these are polyglutamated derivatives that function as co-enzymes in metabolic reactions of the folate cycle providing carbon groups for the production of purine rings. (Wagner 1995). The folate metabolic cycle is also needed for production of thymidylate and remethylation of homocysteine to methionine (Molloy and Scott 2001). At the same time, methionine is critical for the synthesis of the methyl donor S-adenosylmethionine (SAM), which is involved in key methylation reactions including DNA, RNA and protein methylation (Shane 1995 and Anguera et al. 2006).

Deficiencies in folate have been associated with neural tube defects and neurological disorders in children due to impaired biosynthesis of SAM, dTMP and methionine, leading to DNA hypomethylation (Suh et al. 2001). These facts highlight the importance of keeping intracellular and extracellular folate levels for an adequate DNA biosynthesis and expression. Folate levels depend on folate transport, bioavailability, polyglutamation state, cell turnover and folate binding protein concentration (Suh et al. 2001 and Anguera et al. 2006). In view of our results, we propose that the folate binding protein and enzyme FDH may have a key role in maintaining cellular folate concentration, binding to folates such as 5mTHF, the most abundant folate in CSF, and storing it in situations of 5mTHF excess.

This view concurs with the discoveries of Champion et al. (1994) describing FDH knock-out rodents as unable to re-establish their normal extracellular and intracellular folate levels, in spite of being supplemented with folates.

The present study reveals a close association between folate imbalance (raised levels of extracellular 5mTHF) and abnormal arachnoid tissue growth. Thus, we suggest that the positive correlation observed in our "in vitro" experiments between arachnoid cell proliferation and increased 5mTHF is due to a critical dependence of arachnoid tissue on 5mTHF levels in CSF for cell division and differentiation. It is known that 5mTHF levels are high in CSF (Cains et al. 2009) and that folate concentration in CSF is regulated by FDH (Anguera et al. 2006). Therefore, and in accordance with our "in vitro" work, we hypothesize that congenital hydrocephalus is triggered by extracellular 5mTHF accumulation in response to low FDH levels, and consequently folate concentration deregulation, leading to excessive arachnoid cell division. In conclusion, our results imply that cell growth hyperstimulation caused by altered high 5mTHF levels in CSF in congenital hydrocephalus may render the arachnoid tissue unable to exert its functions of controlling and maintaining the CSF out-flow required for the correct development of the fetal brain.

## 3.2 MATERIAL AND METHODS

### 3.2.1 Animals

Animal experiments were performed at the Biological Services Unit at the University of Manchester following the Home Office animal procedures. Rats from the strain of Sprague-Dawley were used and they will be the animal control of choice for future experiments to investigate congenital hydrocephalus. The Sprague- Dawley is suitable for our studies as it has the same period of gestation (21 days) as the rat model for congenital hydrocephalus; the inbred Hydrocephalic Texas strain.

Sprague-Dawley 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 and mouse diet which contains folic acid at 3.2 mg/kg.

### 3.2.2 Matings

Timed matings were arranged to produce age specific fetuses. Generation of timed matings was achieved by housing males and females in the same cage. These cages have a tray attached where vaginal plugs can be found if there is a successful mating. The day on which the vaginal plug is found is considered day zero.

### 3.2.3 Collection of fetal brains and dissection

Fetuses were collected on embryonic day 18. Schedule 1 was performed on designated pregnant rats by specialized technicians in the animal unit. Death was confirmed through a lack of reflexes in eyes and tail. Dissection was always undertaken using sterile tools. The lower abdomen was opened and the uterus taken out and placed on ice to ensure fetal euthanasia. Fetal heads were removed and later soaked in the corresponding culture media.

### 3.2.4 Arachnoid tissue processing and identification

3.2.4.1 Arachnoid tissue processing

Fetal brains were positioned in a dissecting plate using sterile tools and adequate equipment. The fetal arachnoid membrane, a transparent, vascularized and elastic layer situated immediately in contact with the surface of cerebral cortex, was removed and isolated under the dissecting microscope. The membrane was submerged into Dulbecco's modified Eagle's medium (DMEM), (Invitrogen, Paisley, UK) containing 0.05% insulin and 20% FBS (Sigma Aldrich Company, Ltd, Dorset, UK). The leptomeninge was cut into 3 mm fragments and plated in 25-cm$^2$ flasks (Corning Ltd, UK) with complete medium (DMEM with supplements). The flasks were kept at 37°C in an incubator with 5% $CO_2$ atmosphere. The medium was replaced every 2 days. Arachnoid cells were transferred after 72 hours from the bottom of the flask to 24 well plates containing a 10 mm coverslip/well. The media was removed from flasks, and cells were washed with Hanks' buffer and digested in 1:1 (v/v) dilution with 0.25 % (w/v) trypsin and 0.02 (w/v) ethylenediamine tetra-acetic acid (EDTA) for 15 minutes. Complete media was added to stop digestion, and

arachnoid cells were suspended and then mechanically separated and triturated by up and down pipette aspiration. Dissociated cells were counted using a haemocytometer in the presence of 50% Trypan blue (Sigma Aldrich Company Ltd, Dorset, UK) to ensure that the cells to be cultured were all viable. The cell suspension was diluted to achieve a cell concentration of $4 \times 10^5$ cells/ml, and was used for subcultivation in 24 well plates with each well containing 100 μl of cell suspension.

### 3.2.4.2 Arachnoid cell-type identification

Cytokeratin 18 and zone occludens (ZO-1) are cell markers of arachnoid cells, as previously described in published protocols for identification of mammal arachnoid cells (Xin et al. 2010). Tissue isolated from the brain was cut into 3 mm fragments and fixed with 4% paraformaldehyde in PBS. The cells were permeabilized with O.5% TritonX-100 also in PBS for 5-7 minutes, and washed 3 x 5 minutes with a washing solution containing 10% FBS and 0.2% Tween-20 in PBS, before application of primary antibodies. Double staining with mouse anti-human Cytokeratin 18 (Abcam, Cambridge, UK) and rabbit anti-human ZO-1 (Invitrogen, Paisley, UK) was performed sequentially as it is required when using species-specific primary antibodies. Cells were incubated with primary antibodies for 1 hour, washed with washing solution, and incubated with secondary antibodies Alexa Fluor 488 conjugated anti-rabbit or Alexa Fluor 594 anti-mouse (Invitrogen, Paisley, UK) at 1:500 dilution for 1 hour. Finally, cells were washed with washing solution, counterstained with 4'6-diamidino-2-phenylindole (DAPI), and mounted with antifade reagent (Prolong Gold, Invitrogen, Paisley, UK). Images from immunostained arachnoid cells were captured at 300 milliseconds exposure on a Leica DMLB fluorescence microscope connected to a Coolsnap digital camera.

### 3.2.5 **Primary arachnoid cell culture treated with folates and FDH**

Primary arachnoid cell culture were maintained at 37 °C in 5% carbon dioxide for 48 hours to study the cells' response to media containing 5mTHF, THF and folic acid at concentration range ($10^{-4}$-$10^{-9}$ M), with and without inactive ALDHL1 (point mutation in intermediate domain) and active FDH (intact intermediate domain) at constant concentration ($10^{-5}$ M). The FDH concentration range was chosen according to the estimated value obtained in previous Western blots studies for FDH in normal rat CSF (Cain et al. 2009). Inactive and active FDH was kindly provided by Dr. S Krupenco, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC. Inactive FDH was used to study exclusively its folate binding properties in isolation of its enzymatic function. Hence, only the tight bind N domain was active to bind to folates (Fu et al. 1999). Media without folic acid was used as negative control. Cell proliferation was measured using the fluorescent PrestoBlue cell viability reagent kit following the company's protocol (Invitrogen, Paisley, UK). Briefly, PrestoBlue reagent was 1/10 diluted in deionized water and 10µl of solution was added to 100µl media per well. This step was followed by 30 minutes incubation at 37°C. Fluorescence signals due to enzymatic activity, and therefore cell division, were measured on a Magellan 200 fluorimeter (TECAN, UK), set up at 535 nm and 615 nm for wavelength excitation and emission respectively. All folate solutions were purged with Argon before being added to the arachnoid media in order to avoid folates oxidation, which would render folates unstable and inactive.

### 3.2.6 DAPI staining of primary arachnoid cell cultures treated with folates and FDH

DAPI staining was performed for imaging and visualization of cultured arachnoid cells exposed to FDH and folates. Blue-fluorescent DAPI nucleic acid stain (Invitrogen, Paisley UK) was carried out following the company's instructions. Cells plated in poly-D lysine-coated 24-well plates containing 10 mm glass round coverslips (VWR International, Ltd.), and cells attached at an initial density of $4 \times 10^5$ cells/ml, were washed with washing solution, counterstained with 4'6-diamidino-2-phenylindole (DAPI) and mounted with antifade reagent (Prolong Gold, Invitrogen, Paisley, UK). Images from DAPI stained arachnoid cells were captured at 300 milliseconds exposure on a Leica DMLB fluorescence microscope connected to a Coolsnap digital camera. DAPI stains dsDNA preferentially, giving rise to fluorescence enhancement that can be detected with a violet filter.

### 3.2.7 CSF treatment

After 48h growth, cells in the 24 well plates were treated with 50% normal and abnormal CSF diluted in complete media. After treatment, the cells were left to grow for another 24h in order to investigate their effect on cell proliferation. Cell growth was determined using the colorimetric assay MTT (Tetrazolium dye cell viability kit (R& D Systems, UK). This assay assesses cell metabolic activity by measuring changes in color (optical density) after reduction of MTT by enzymes which turn the yellow MTT dye into a purple color. Optical density is directly proportional to the number of viable cells in the media.

3.2.8 **Statistical analysis**

Cell culture experimental data from the effect of folate and FDH supplementation on cell proliferation was analysed by two-way ANOVA. In regard to CSF, its impact on cell proliferation, as well as its effect on cell death was assessed by one-way ANOVA.

## 3.3 RESULTS

### 3.3.1 Immunocytological assessment
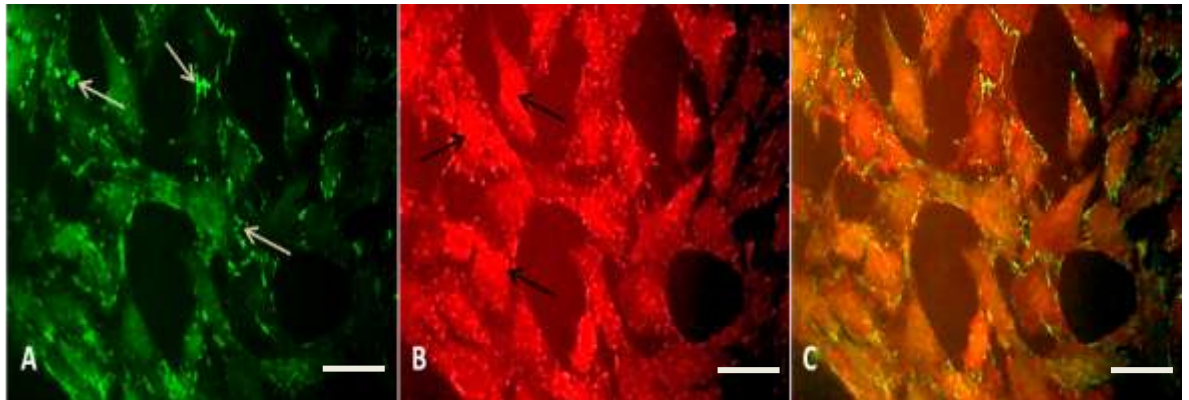


**Figure 3.1. Fluorescent immunohistochemical staining of arachnoid tissue at embryonic day 18. The staining shows mesenquimal polygonal cells linked together by junctions (zone occludens-1) identified as green fluorescence and pointed with white arrows in image A. Staining of cell marker cytokeratin 18 in cytoplasm (fluorescent red, black arrows) is shown in image B. Image C represents A and B merging, N=6, scale bar: 50 μm.**

Fetal meningeal arachnoid tissue was isolated and attached to fibronectin-coated coverslips. Arachnoid cell phenotype was confirmed and characterized by using arachnoid-specific primary antibodies to detect junction arachnoid cell markers (Zone occludens-1 (ZO-1)) and cytoplasmic-specific protein (Cytokeratin 18). Primary antibody expression was visualized by labelling the cells with secondary antibodies conjugated to fluorescent molecules. Secondary antibodies were anti-IgG FITC (green) for localization of ZO-1 and Alexa fluor 555 (red) for identification of cytokeratin 8.  Fetal arachnoid cells exhibited a polygonal shape with junctions or ZO-1 (green staining) between cells (Figure 3.1, A). The cells' cytoskeleton presented dense clusters of arachnoid-specific cytokeratin which was revealed with red fluorescence (B, black arrows). Images A and B where merged (C) to visualize location of ZO-1 junctions in relation to cytokeratin 18.  Once arachnoid tissue was confirmed by specific cell markers, and the tissue was "in vitro" cultured for production of subsequent primary cell cultures to be used for folate effect studies.

3.3.2 **Morphological and confluency assessment**
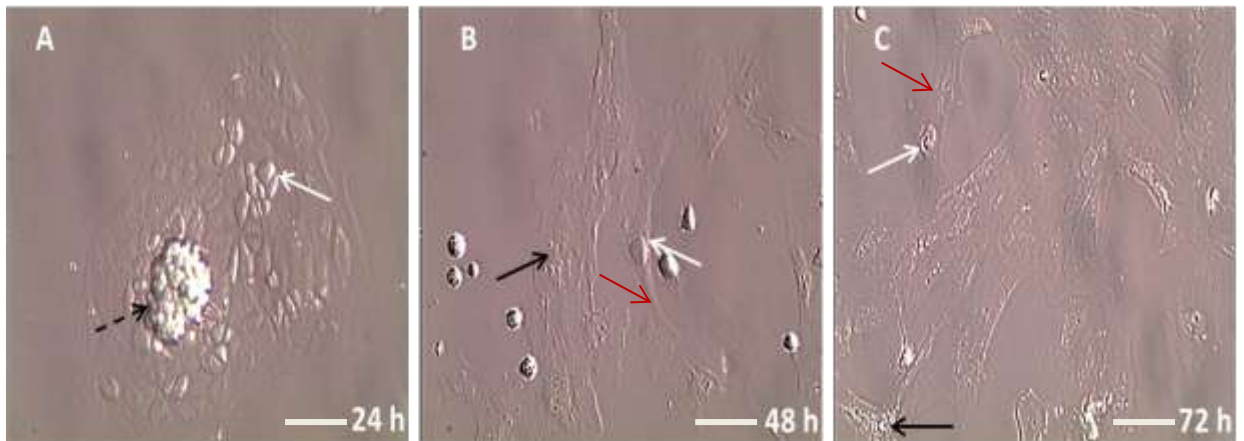


**Figure 3.2 Cultured arachnoid cells micrographs using bright-field microscopy show cell appearance and confluence after 24 hours (A), 48 hours (B) and 72 hours (C), N:6, scale bar: 50 μm.**

Fetal arachnoid fragments (Figure 3.2, A, dashed black arrow) were used for production of primary cell cultures. These attached to the bottom of flasks creating tissue-like structures or scaffolds distributed as a monolayer of polygonal cells with large nuclei (Figure. 3, A, B and C, white arrows) and many granules in cytoplasm (Figure. 3 B and C, black arrows). Also, the cells attach together forming sheets that bridge together (Figure. 3 B and C, red arrows). This agrees with characteristics described for this cell-type in previous publications (Murphy et al. 1991). Cell proliferation increased progressively at 24 hours growth to achieve total confluence after 72 hours.

### 3.3.3 FDH is counterproductive for arachnoidal cell growth in the presence of 5mTHF.
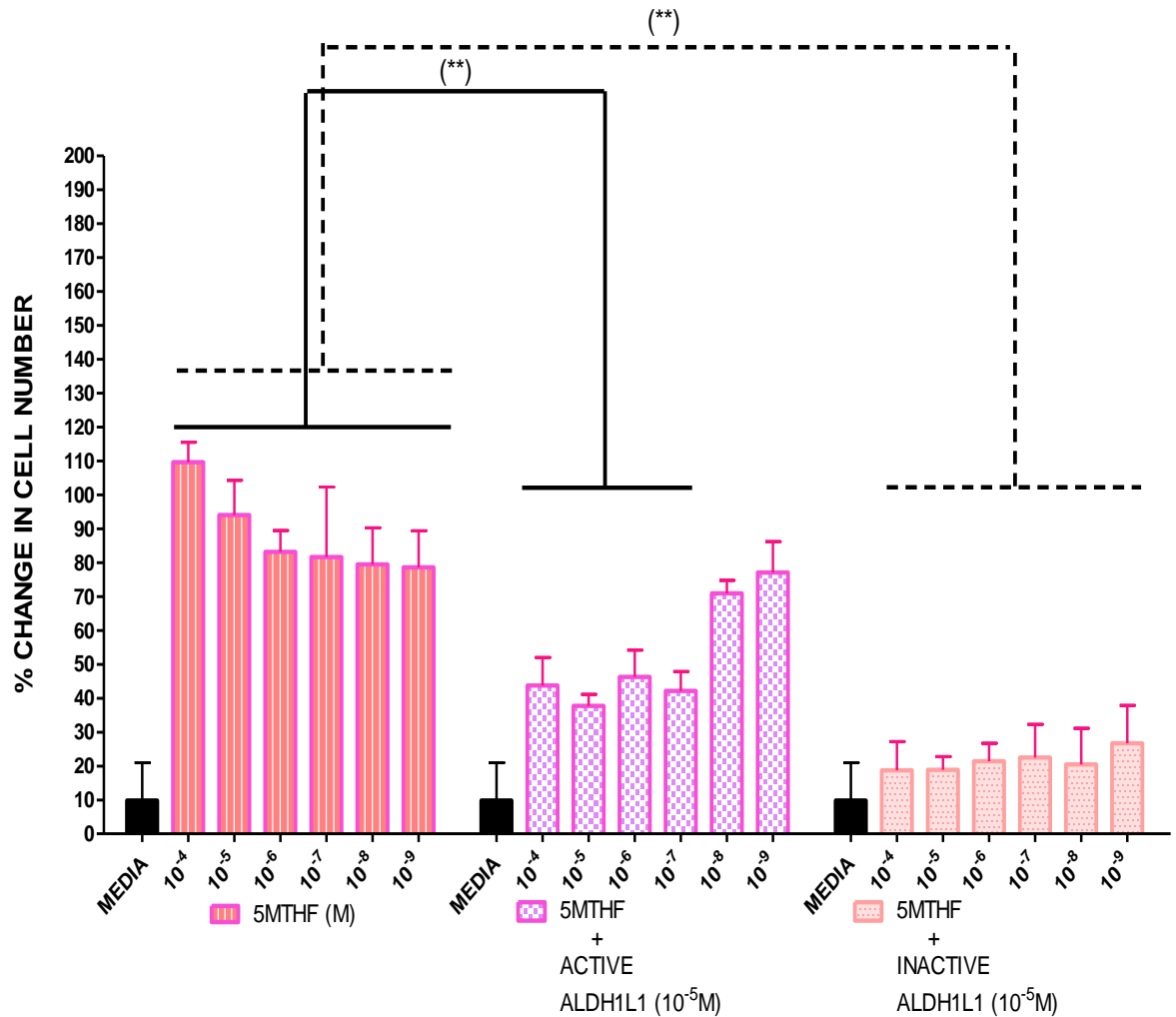


**Figure 3.3. Effect of decreasing 5mTHF and constant active and inactive FDH/ALDH1L1 concentration on cell proliferation. MTHF alone (line pattern), 5mTHF + active FDH (square pattern) and 5mTHF + inactive FDH (dot pattern). Arachnoid cells from E19 Sprague- Dawley foetuses were incubated with metabolites in the range $10^{-4}$ - $10^{-9}$M in the absence and presence of FDH at $10^{-5}$ M. Cell bioavailability was measured using a fluorescence-based assay as described in material and methods (paragraph 3.2.5). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of variance with post hoc test revealed significant differences in the indicated paired samples. Results are shown as: \*\*p < 0.01 for the effect on proliferation of 5mTHF alone versus 5mTHF in combination with FDH.**

To assess the effect of constant FDH and decreasing 5mTHF concentration on cell growth, the metabolite was added to the arachnoid media at concentration range ($10^{-4}$- $10^{-9}$ M) with and without active and inactive FDH at $10^{-5}$ M (Figure 3.3). Overall, control experiments showed that E18 arachnoid cell cultured in folate supplemented DMEM media showed an increase in cell number above media control levels (Figure 3.3, media columns; no folates/black columns) after 72 hours growth. Thus, the average percentage increases were of $87.80 \pm 12.07$, $52.98 \pm 16.62$ and $21.52 \pm 2.86$ for media containing 5mTHF alone (Figure 3.3, first set of columns), 5mTHF plus active FDH (Figure 3.3, second set of columns) and 5mTHF plus inactive FDH (Figure 3.3, third set of columns). Interestingly, media containing just 5mTHF cell growth was exacerbated at all concentrations and peaked at $10^{-4}$ M with percentage increases in cell number as follows: 109.63 ($10^{-4}$ M), 94.2 ($10^{-5}$), 83.19 ($10^{-6}$), 81.69 ($10^{-7}$), 79.49 ($10^{-8}$), 78.61 ($10^{-9}$). These data clearly demonstrate that cell proliferation was directly proportional to the concentration of 5mTHF in the media. In contrast, the presence of FDH in combination with 5mTHF had a significant detrimental effect on cell growth with both active and inactive protein ($p < 0.01$).

In conclusion, these data confirm that the addition of active FDH in the folate (5mTHF) supplemented media caused significative decreases in growth percentage with cell cultures presenting a reduction in cell proliferation of 65.89% ($10^{-4}$ M), 56.47% ($10^{-5}$ M), 36.93% ($10^{-6}$ M), 39.51% ($10^{-7}$ M), 8.54% ($10^{-8}$ M) and 2.55% ($10^{-9}$ M) when compared to media containing 5mTHF alone. This reduction was significantly more pronounced in the presence of inactive FDH ($p < 0.01$), with decreases in growth as follows: 90.91% ($10^{-4}$ M), 74.8% ($10^{-5}$ M), 60.79% ($10^{-6}$ M), 60.19% ($10^{-7}$ M), 59.04% ($10^{-8}$ M) and 51.92% ($10^{-9}$ M). These data indicate that FDH has a generalized negative effect on cell growth in the presence of 5mTHF, which correlates with decreasing 5mTHF concentration in the media.

Interestingly, with active FDH, the negative impact on cell growth is less apparent when 5mTHF was at $10^{-8}$ M and $10^{-9}$ M, indicating that FDH effects on growth depends on its specific concentration in the media.

### 3.3.4 THF with or without FDH does not potentiate cell arachnoid growth.
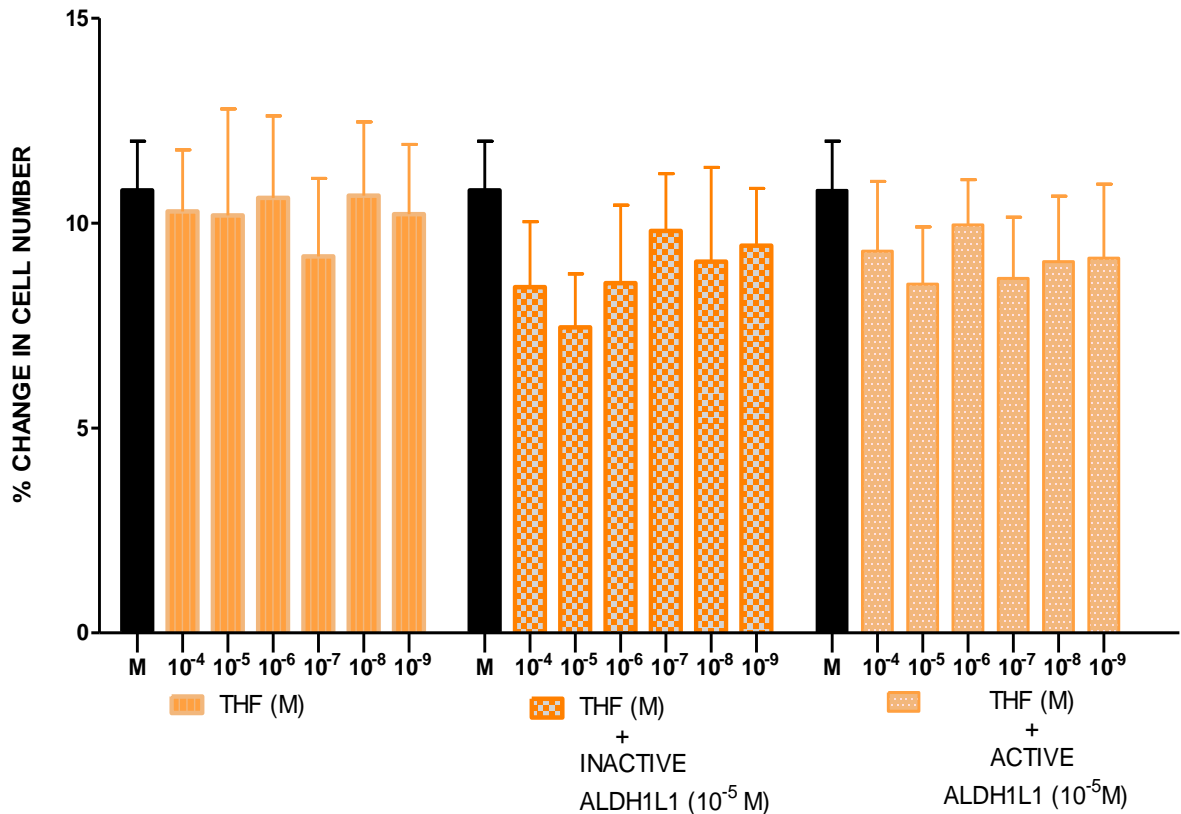


**Figure 3.4. Effect on cell proliferation of THF alone (line pattern), THF plus inactive ALDHL1 (FDH) (square pattern) and THF plus active ALDH1L1 (FDH) (dot pattern) at decreasing concentrations. Cell bioavailability was measured using a fluorescence-based assay as described in material and methods (paragraph 3.2.5). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of data with Kruskas-Wallis test and post-test Dunn's multiple column comparison revealed THF had no effect on cell proliferation with or without ALDH1L1 (FDH).**

FDH has a high affinity for THF, a product of its enzymatic reaction. Taking this fact into consideration, the effect of both molecules on cell division was determined by adding to arachnoid media THF alone, THF in combination with FDH and finally THF with active FDH. Cells in all conditions had a growth percentage below media control levels (M) (10.8%) after 72 hours growth (Figure 3.4). This reduction in growth was relatively less intense in arachnoid cells co-cultured with inactive FDH and THF, when compared to

growth in the presence of THF alone, with an averaged reduction in growth of 8.7% and 10.1% respectively (Figure 3.4, second set of columns). With regard to active FDH and 5mTHF, the average reduction in percentage cell growth values was 9.06%, which was also lower than controls (M) in Figure 3.4, (third set of graphs). In conclusion, these data indicate that THF alone, as well as along with FDH in the media, may not be beneficial for arachnoid cells, although these data were not statistically significant ($p \geq 0.05$).

### 3.3.5 Folic acid exacerbates growth with and without FDH
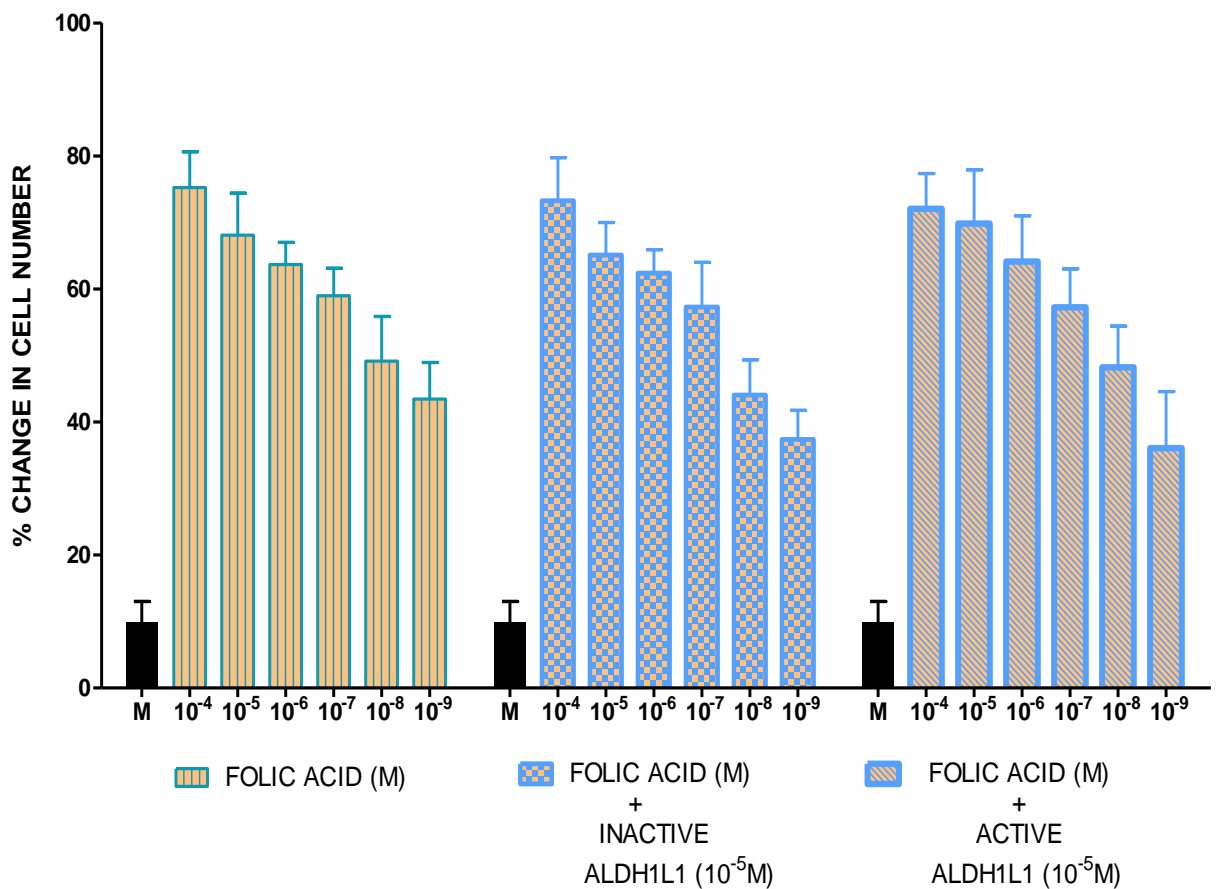


**Figure 3.5. Effect on cell proliferation of folic acid alone (line pattern), folic acid plus inactive ALDH1L1 (FDH), (square pattern) and folic acid plus active ALDH1L1 (FDH), (dot pattern) at decreasing concentrations. Cell bioavailability was measured using a fluorescence-based assay as described in material and methods (3.2.5). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of data with Kruskas-Wallis test and post-test Dunn's multiple column comparison revealed FDH did not have a positive effect on cell growth and the average cell growth was similar at all conditions in the presence of folic acid with and without FDH**

Folic acid is a biosynthetically artificially produced folate, which is normally present in commercial media. For this reason, only DMEM media depleted of folic acid was used as the control media throughout all experiments. Folic acid was added to DMEM media to study its effect on cell division, which was subsequently assessed by comparison with media control (no folates). Folic acid's effect on cell division was also determined in the presence and absence of inactive and active FDH. Figure 3.5 shows that cells in all conditions presented a similar average percentage increase above media control levels of $59.77 \pm 11.86$ for folic acid alone, $57.93 \pm 13.5$ for folic acid + inactive FDH and $56.58 \pm 13.7$ for folic acid + active ALDH1L. In conclusion, FDH does not significantly interfere with cell growth in the presence of folic acid and cell division remains similar to media containing folic acid only ($p > 0.05$).

### 3.3.6 **FDH effect on arachnoidal cell growth depends on folate presence in media**
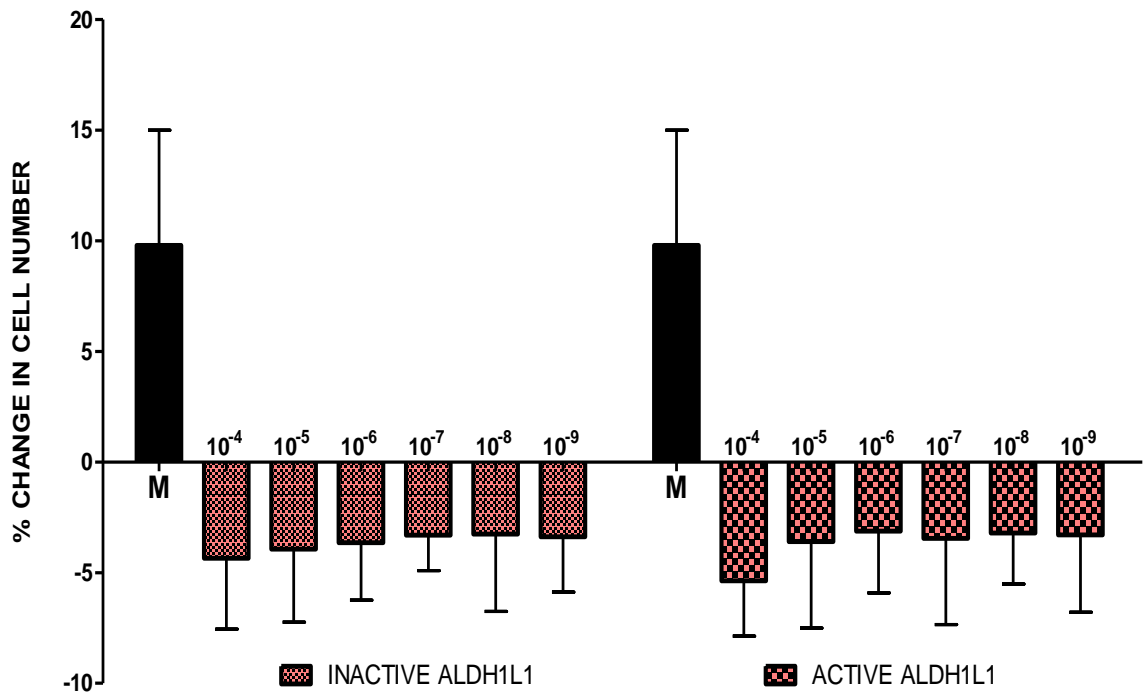


**Figure 3.6. Effect on cell proliferation of active (small square) and inactive FDH (large square). Proliferation was measured using a fluorescence-based assay as described in material and methods (3.2.5). Data are presented as mean percentages in cell change ± SEM obtained after normalization of relative fluorescence indexes (RFI) measurements (n=6). Analysis of data with Kruskas-Wallis test and post-test Dunn's multiple column comparison revealed no change in cell growth either with inactive or active FDH.**

In order to study whether there is any effect of active and inactive FDH alone on arachnoid cells, the folate binding protein was added to the media without any folates. Arachnoid cells cannot survive without folates, and as expected, addition of single FDH to a media depleted of folates resulted in an overall negative growth at all concentrations (Figure 3.6 above).

3.3.7 **DAPI staining reveals different effects on growth of arachnoidal cells co-cultured with folates and FDH.**

DAPI staining was performed on cell cultures treated with folates and FDH to study their effect on cell proliferation visually. As expected, these results agreed with fluorescence quantification analysis of cell division after treatment with folates and FDH using the fluorescence based assay (Presto Blue), (paragraph 3.2.6). DAPI staining results in the presence of specific folates at $10^{-5}$ M with and without FDH also at $10^{-5}$ M are illustrated in Figure 3.7 below:
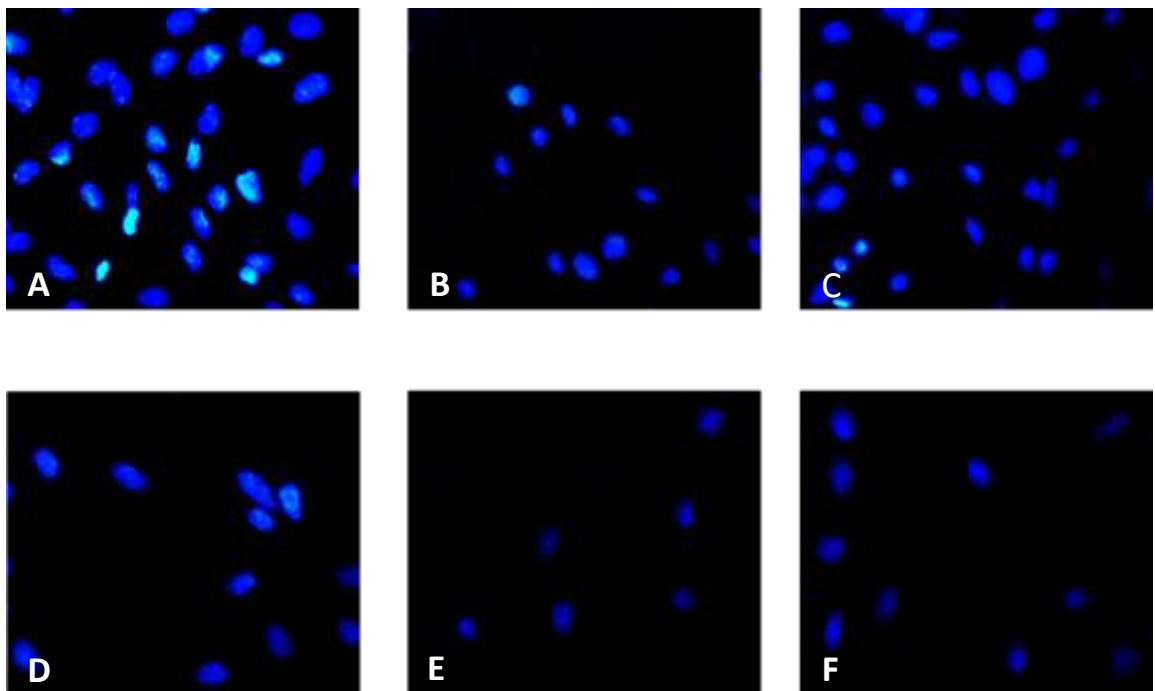


**Figure 3.7. DAPI nuclei staining snapshots of primary cell cultures in the presence of different types of folates with and without FDH as follows: 5mTHF (A), 5mTHF + ACTIVE FDH (B), FOLIC ACID (C), THF(D), THF + ACTIVE FDH (E) and negative control (NO FOLATES), (F), (x20) magnification.**

Figure. 3.7 shows how 5mTHF potentiates cell division (A), whereas the presence of FDH results in a negative effect on cell growth (B). In a similar fashion to 5mTHF, folic acid also encouraged cell proliferation (C). On the other hand, growth with THF had a negative impact on cell division with and without FDH (D) and (F) respectively, with cell growth similar to negative controls. This suggested a halt in cell division in the presence of THF.

### 3.3.8. **CSF effect on arachnoid cell growth**

In past studies, normal CSF was shown to have a beneficial effect on cell growth of cerebral cortex cells, whilst hydrocephalic CSF was fond to be detrimental. In order to investigate whether there is a role for CSF in the proliferation and development of arachnoid cells, this body fluid was placed in arachnoid media depleted of folates and FDH. After 24 hours growth with arachnoid media enriched at 50% CSF, cell bioavailability was measured using a colorimetric assay (MTT assay) as described in material and methods. Optical density was quantified, and this data was used to calculate percentage cell changes with and without CSF. Percentage cell increases are represented versus arachnoid samples, as shown in Figure 3.8 below:
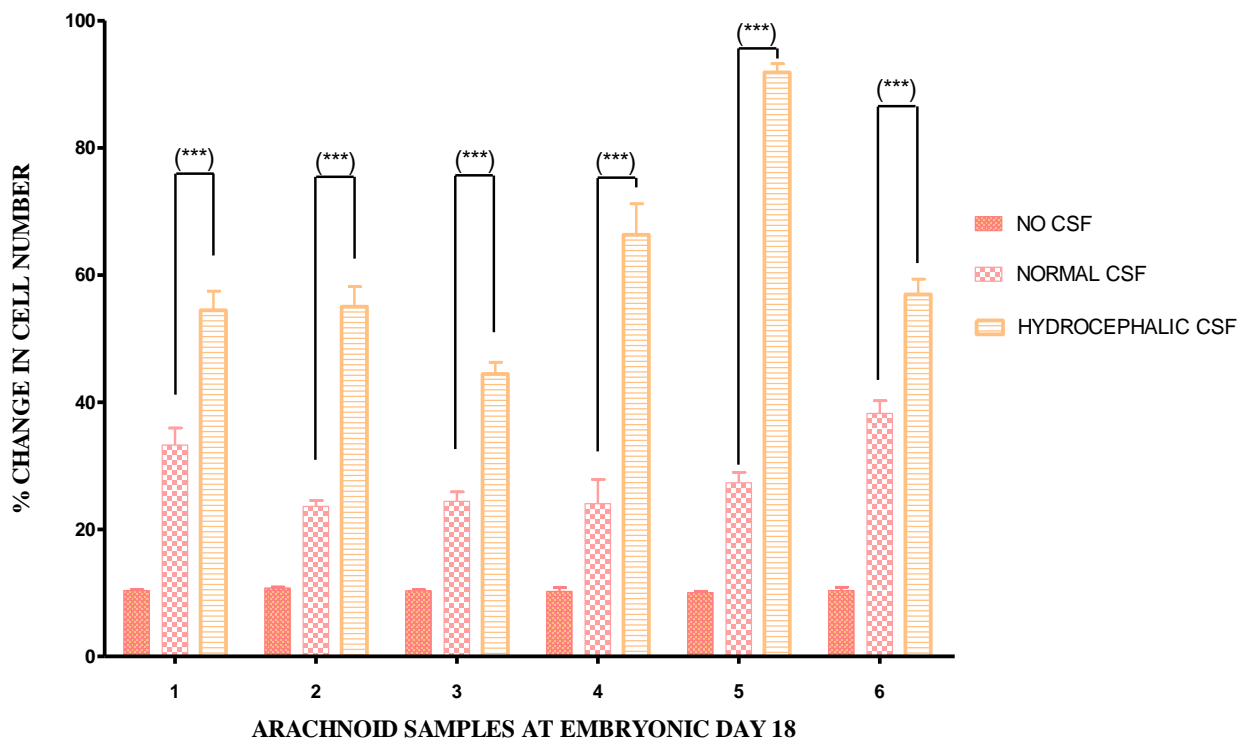


**Figure 3.8. CSF effect on arachnoid cell proliferation. Dot bars represent CSF-untreated cultures, cultures enriched with normal 50% CSF (squares) and cultures enriched with 50% hydrocephalic CSF (horizontal lines). Analysis was performed on six different arachnoid samples. Data are presented as mean percentages in cell change ± SEM obtained after normalization of optical density measurements. Analysis of variance (two-ways ANOVA) with Bonferroni post hoc test revealed significant differences in all indicated paired samples, N=6. Results are shown as: ***p < 0.001 for the effect on proliferation of normal CSF versus hydrocephalic CSF.**

Figure 3.8 shows that the presence of normal and hydrocephalic CSF clearly encouraged arachnoid cell division when compared to media devoided of CSF. Surprisingly, this outcome contradicted the results of previous published research using cerebral cortex cells, which after exposure to hydrocephalic CSF resulted in cell arrest in the S phase of the cell cycle. In this study, all samples exposed to normal CSF show an average 17.77% increase in cell growth with respect to media without CSF. With regard to samples treated with hydrocephalic CSF, the average percentage increase was 50.78% when compared to untreated samples. This percentage increase also meant that hydrocephalic CSF stimulated growth even more than normal CSF, with 33% more growth due to the presence of abnormal CSF in the media after 24 hours ($p < 0.001$). Finally, figure 3.8 also shows variability between samples with the same treatment. For instance, sample 5 and sample 3, which were both treated with hydrocephalic CSF, presented very different percentage increases, with the sharpest percentage increase (92.2%) for sample 5 in contrast with sample 3 (46.56%). A more similar trend in percentage change was shown by samples treated with normal CSF, with sample 6 presenting the highest percentage increase (35.9%) and sample 1 the lowest (22.5%).

## 3.4 CONCLUSION-DISCUSSION

The adequate development of fetal arachnoid tissue is crucial for cerebrospinal fluid egress and the failure of that function leads to fluid build-up and congenital hydrocephalus (Cornelius et al. 2011). In this study, morphological and immunocytological assessment allowed characterization of cytoskeletal protein expression and intercellular junctional proteins typical in tissues with a function in fluid flow regulation (Bhavya et al. 2007). Morphological and immunocytological assessment were also critical in order to confirm arachnoid tissue phenotype for later production of primary cell cultures, and to ultimately determine the effects of folate, and normal and abnormal CSF on arachnoid cell growth.

In previously published studies (Cain et al. 2009), folates such as THF, in combination with folinic acid, encouraged cerebral cortex growth and differentiation "in vivo" and "in vitro". In this study, folates' effect on cell division was also assessed, but on this occasion on arachnoid primary cell cultures. Results from this investigation revealed 5mTHF as the only folate able to potentiate cell arachnoid division and subsequent production of tissue-like sheets that distribute as monolayer of polygonal cells at the bottom of flasks. These characteristics are typical in arachnoid cell growth "in vitro", and meant that 5mTHF contributes to the production of cell to cell connections, allowing flow regulation. This outcome also indicates that 5mTHF is the key folate for arachnoid cell growth, and suggests that this folate may be also crucial for the correct development of a functional fetal arachnoid in early pregnancy. In contrast, 5mTHF co-culture with active FDH was counterproductive for cell growth at specific concentrations of 5mTHF and FDH. For instance, when 5mTHF was present at concentration range ($10^{-4}$–$10^{-9}$ M) and active FDH at concentration present in normal CSF ($10^{-5}$ M), ( $p \leq 0.05$).

FDH binds to 5mTHF and limits arachnoid cells' capability to use the folate for cell division. This finding also agrees with results from experiments with cerebral cortex cells cultured with 5mTHF and FDH where cell division was also negatively affected by the presence of FDH in the media at pre-determined concentrations. In contrast, active FDH at the same concentration ($10^{-5}$ M) and 5mTHF at small concentrations ($10^{-8}$-$10^{-9}$ M) potentiated cell growth. However, this result was non-statistically significant, and we argue that a more extensive dose response study is needed to clarify this result. Nevertheless, once more, our experiments suggest that FDH is a folate binding protein responsible for folate regulatory function in CSF, making it available to cells or sequestering it instead depending on 5mTHF specific concentration in CSF. Our findings also agree with the research of Anguera et al. (2006) describing FDH as an enzyme that is able to pool folates in situations of high folate concentration. In addition, it is known that folates like 5mTHF can be trapped in situations of cobalamine deficiency (Krumdieck et al. 1978) impeding its conversion into THF. This is important, as THF is a key folate that cells need for DNA methylation and DNA synthesis in the folate metabolic cycle. THF is also needed for the production of S-Adenosyl-L- methionine (SAM) a common substrate involved in methyl group transfers and crucial for more than 100 metabolic reactions in the body (Roje, 2006).

In the hydrocephalic brain, CSF lacks FDH, which suggests that folate concentration in CSF is not regulated by the enzyme. Therefore, 5mTHF may be accumulated in the body fluid, leading downstream to an exacerbation of arachnoid tissue proliferation, as observed in our "in vitro" experiments in situations of high levels of 5mTHF in the presence of active FDH. Our experiments also showed that arachnoid primary cell culture treatment with hydrocephalic CSF stimulates arachnoid cell division, and this effect was much more noticeable when contrasting cell cultures treated with hydrocephalic CSF with cell cultures

without CSF or cultures with just normal CSF. These findings agreed with past studies on analysis of hydrocephalic CSF, which showed higher levels of 5mTHF in hydrocephalic CSF than normal CSF (Cains et al. 1989). Our results also agree with Gonzalez et al. experiments in 2006, describing CSF from hydrocephalic rats as a promoter of neuronal and glial differentiation.

Whilst brain alterations in congenital hydrocephalus are mainly related to brain loss, there are also mechanisms by which the hydrocephalic brain compensates for brain damage by repairing with exacerbated cell proliferation. For instance, certain subpopulations of astrocytes respond to neuroepithelia denudation by increasing the cell division rate and by creating ependymal-like new barriers in the affected areas (Rodriguez et al. 2006). Hereby, our studies suggest that the arachnoid tissue responds to hydrocephalic CSF and its changes in composition (high levels of 5mTHF and low levels of FDH) by encouraging cell division.

In conclusion, we propose that FDH is a regulatory folate binding protein, and its depletion in hydrocephalic CSF is the cause of increased 5mTHF levels leading to abnormal arachnoid development and dysfunction.

Furthermore, it is known that a lack of FRα (5mTHF transporter) in the brain, leads to brain deficits (Steinfeld et al. 2009). Similar to FRα, FDH is a folate binding protein in brain CSF that is able to bind to 5mTHF. Consequently, its absence in CSF may account

not only for congenital hydrocephalus but also for other fetal brain disorders related to 5mTHF deficit in CSF.

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# CHAPTER 4

# INGENUITY PATH ANALYSIS OF NORMAL VERSUS HYDROCEPHALIC CSF'S PROTEIN PROFILES IDENTIFIED BY LCMS.

**ABSTRACT:**

Congenital hydrocephalus is triggered during the first trimester of pregnancy and begins with increased intracranial pressure due to cerebrospinal fluid (CSF) build-up. This is followed by variations in CSF constituents related to the folate metabolic cycle, and reduced thickness of the cortical plate. These events result in mental retardation, intellectual disability and abnormal CSF proteome.

In this investigation, LCMS analysis was performed to identify differences in CSF protein profiles expressed during normal and hydrocephalic conditions, focusing on the presence and absence of proteins in normal and abnormal CSF. Variations in LCMS protein profiles were analysed, first using Microsoft Excel to filter LCMS data and select the proteins unique to each type of CSF, and subsequently by a second analysis of Excel datasets using a bioinformatic tool called Ingenuity Path Analysis (IPA). IPA allows protein matching with metabolic pathways exclusively represented in hydrocephalic CSF and missing in normal CSF, and vice versa. The ultimate aim is to help in the discovery of key metabolites, which are either missing or in excess in hydrocephalic CSF, that may be good candidates for the development of novel therapies. Furthermore, the present study may give some insights into the pathology of congenital hydrocephalus, which has not yet been fully characterized, and open new avenues for an alternative treatment to shunting, the current treatment, which is associated with a high risk of infection and technical failures.

## 4.1 INTRODUCTION

Brain CSF is a nutritional fluid vital for CSN development. It is secreted by the choroid plexuses in the brain ventricles where it accumulates if bulk flow is interrupted. CSF obstruction is caused either by obstruction of the cerebral aqueduct or by abnormal absorption within the arachnoidal villi in contact with the general blood circulation where CSF is drained. CSF blockage leads to enlarge ventricles also called hydrocephalus (water in the brain). Congenital hydrocephalus begin early in pregnancy and starts with increased intracranial pressure caused by CSF accumulation leading to intellectual impairment as well as CSF compositional changes. These events suggest that normal and hydrocephalic CSF presents a differential protein spectrum. Based on this assumption, past studies relied on LCMS analysis (Nabiuni et al. 2006) to identify the specific protein profile corresponding to normal and abnormal CSF. LCMS is a powerful analytical technique used in the clinical diagnosis of neurological and metabolic disorders (Grebe et al. 2011, Johnson et al. 2005). The technique combines physical separation of chemical molecules by liquid chromatography (LC) with mass analysis by mass spectrophotometry (MS) of proteins previously fragmented by enzymatic digestion (Glish and Vacher, 2003). LC generates a peptide fragmentation pattern, and MS allows sensitive peptide detection based on molecular mass and particle charge once peptides are ionized with electron beams that render the peptides positively charged. After ionization, molecules are separated according to their mass-to-charge ratio by means of electromagnetic fields in a built-in analyser. MS includes ion detectors that allow protein identification at the same time as protein quantification. The resulting MS data can be further analysed with Mascot software, a database that further identifies proteins according to their peptides' molecular weight patterns and amino acid sequences. The end result is a list of proteins with an assigned

accession number and corresponding chemical names (Arpino, 1992, Glish and Vacher, 2003).

In past investigations, we examined the CSF protein profile in normal and hydrocephalic fetal rodents using LCMS followed by Mascot software for identification of proteins related to the folate metabolic cycle. Deficiencies in folate enzymes and folate transporters in the brain are linked to neural tube defects and severe developmental regression (Antony, 2007 and Steinfeld et al. 2009). In our previous study, two lists of proteins were generated by LCMS, one corresponding to normal CSF and a second one corresponding to abnormal CSF. The LCMS protein profile analysis gave evidence of differences in CSF composition between normal and hydrocephalic CSF. These differences were clear in terms of folate enzymes where hydrocephalic CSF appeared to be devoid of the enzyme formyl-tetrahydrofolate dehydrogenase, a key enzyme in the folate metabolic cycle involved in the production of tetrahydrofolate (THF) from formyl-tetrahydrofolate. THF is crucial in brain development as this co-enzyme, which is at the core of the folate metabolic cycle, is responsible for the synthesis of the methyl donor S-adenosyl-L-methionine (SAM). SAM is responsible for more than 100 metabolic reactions in the body and it is crucial for DNA methylation and production of methionine (Mischoulon and Maurizio, 2002); hence the importance of maintaining normal concentrations of FDH in CSF for the correct development of the fetal brain.

In the present study, we focus not only on folate enzymes but on the global protein profile obtained previously from LCMS analysis of CSF. Thus, all LCMS datasets were first grouped using the Microsoft Excel in order to filter the information and to identify differences between normal and hydrocephalic CSF. Each LCMS listed protein

corresponding to normal and hydrocephalic CSF respectively was allocated an identification number used for later comparison searches in the LCMS lists. Normal protein identification numbers which were missing in abnormal CSF were assigned a specific value that was later captured by means of the Excel tool autofilter. The Excel analysis resulted in two protein profiles, one representing exclusively proteins found in normal CSF, and a second list representing the proteins exclusively found in hydrocephalic CSF. Proteins common to normal and hydrocephalic CSF were excluded. In the present study we also carried out a further analysis of LCMS protein profiles utilizing a bioinformatic tool called Ingenuity Path Analysis (IPA). IPA creates associations between the proteins identified by LCMS and the metabolic pathways where related proteins may fall.

We speculate that proteins present in abnormal CSF but missing in normal CSF may be potential biomarkers of congenital hydrocephalus. Thus, a comparative analysis was performed in order to determine the specific metabolic pathways represented in the normal CSF and abnormal CSF. IPA differential metabolic pathway identification for normal and hydrocephalic CSF implied, in our opinion, the activation of genes involved in the metabolic pathways found by IPA, and downregulation of the genes that passed undetected.

IPA analysis is based on p-value measurements for a given process. These are calculated taking into account the specific number of genes found in our dataset, which are known to be linked to that process, and a reference total set of genes recognized by IPA databases as part of biological processes associated to the specific canonical pathway (www.ingenuity.com). Therefore, on this occasion, the p-value is a measure of the

probability that the IPA relationships between LCMS focus genes and a given metabolic pathway or biological process is due only to random chance.

In conclusion, we performed IPA analysis to identify genes which are not represented in hydrocephalic CSF, as they may be indicative of inactivation of specific metabolic routes. On the other hand and regarding the genes exclusively detected in hydrocephalic CSF, we propose that they must be activated, thus explaining their absence in normal CSF. Based on these assumptions, the aim of this study is to discover the canonical pathways and associated biological processes that are suspected to be active in abnormal CSF in contrast with normal CSF, and to shed some light on the complex pathology of a disorder which is still not fully understood. We also intend to draw attention to aspects of the disorder other than the impairment of the folate metabolic cycle and contribute to the identification of biomarkers and novel drugs for a better treatment and prevention.

## 4.2 MATERIAL AND METHODS

4.2.1. **EXCEL analysis**

LCMS protein profile analysis was performed as described by Nabiuni and colleagues in 2009. LCMS protein lists corresponding to normal and abnormal CSF respectively were entered into EXCEL spreadsheets. The analysis started with the assigning of a specific numerical value to each protein within the protein list pertaining to normal CSF (reference list). For instance, protein A was given the value "1" in order to identify it in the list of proteins representing abnormal CSF, if present. Of notice, the assigned number also gives information about order and position in the list. When a protein is missing in the abnormal CSF a, "-1" value is assigned, which means "error". This is achieved by using the "if error" formula. This formula allows the error to be tracked, and a "-1" number will be displayed in the cell. Therefore, proteins that do not match between lists i.e. proteins missing will have the value "-1". The command "autofilter" was also used to select the proteins to be displayed. For example, we can obtain a list with all proteins missing in abnormal CSF by "autofiltering" all cells that contain values other than "-1" in the list of proteins from normal CSF (reference list). Three lists of proteins were obtained using the corresponding "autofilters": list of proteins unique to normal CSF, proteins unique to abnormal CSF and list of common proteins for both normal and abnormal CSF.

4.2.2 **LCMS protein analysis.**

The majority of diseases and biological processes manifest at the protein level. Since there is not always a good correlation between protein and gene expression, protein expression profiles produced by LCMS technology can be useful as an alternative and complementary approach to genomics (Wang 1999, Uriarte 2008, Dai et al. 2009).

Past studies in our laboratory showed that LCMS is a suitable tool for identification of protein sequences in normal and hydrocephalic CSF using Mascot search software for the MS data. This computer program integrates data based on molecular weight and amino acid sequence, and specific fixed modifications (Carbamidomethyl of C) and variable modifications (Oxidation of M) were considered in the Mascot search engine for protein identification. A confidence limit was also chosen based on the peptide fragmentation pattern obtained after previous enzymatic digestion of every single protein, as well as the unequivocal identification of every peptide fragment (Glish and Vachet, 2003). The LCMS data obtained after Mascot search consisted of two lists of proteins, corresponding to normal and abnormal CSF respectively. Each identified protein was accompanied by its equivalent accession numbers and biochemical name (Nabiuni 2006). Both lists of proteins were contrasted, and special attention was given to proteins involved in the folate metabolic cycle and present in normal CSF but absent in abnormal CSF. The logic behind this approach was decided on the basis that folates played a vital role in the development of the cerebral cortex in Texas rats. Past studies, demonstrated how CSF enriched with thymidine (nucleoside), and folates (THF and folinic acid) significantly encouraged cerebral cortex cell proliferation "in vivo" (Cain et al 2009) reducing, hereby, the

incidence of congenital hydrocephalus. On this scenario, this study concentrates on investigating the composition of hydrocephalic CSF in contrast to normal CSF in order to investigate what proteins and associated metabolic pathways are missing in CSF, but present in normal CSF, and vice versa.

4.2.3 **IPA analysis.**

Ingenuity path analysis (IPA) is a bioinformatic tool that makes use of databases containing the knowledge available in the literature about protein and gene information, as well as protein-protein and protein-gene interactions. IPA analysis needs list of proteins with recognizable identifiers or accession numbers, and match the protein identification number with the gene accession number. After protein-gene matching, the IPA tool performs a core analysis to predict what proteins/genes are associated with a specific biological function or disease, and with canonical pathways ([www.ingenuity.com](www.ingenuity.com)) The greater the number of proteins associated to a specific biological function, the greater the possibility of that function being activated. However, the predictive value of the IPA tool is low, and does not give any information about the directionality of the activated process, unless it is accompanied by gene or protein quantification. Nevertheless, protein expression profiling lists produced by LCMS can be uploaded to the IPA server. Lists of proteins can then be core analysed as a starting point in any research that aims to predict the molecular interactions between proteins, and its involvement and association with specific cell functions or diseases (Calvano et al. 2005, Dai et al. 2009).

A global canonical pathway analysis (GCP) was carried out on EXCEL categories (protein lists). P-value measurements obtained from GCP analysis were equivalent to the relationships found between related genes in the EXCEL categories and their associated metabolic pathways or biological processes. The greater the number of genes involved in a pathway or process, the more likely it is that the association between focus genes and metabolic pathway is not due to mere chance. P-values were calculated using the right-tailed Fisher Exact Test, which is already built into the IPA software. P-values less than

0.05 mean results are statistically significant and non-randomly associated. Ratios are also

calculated by IPA, and represent the number of molecules found in a metabolic pathway in

relation to the total number of molecules included in the metabolic pathway. A threshold

ratio $\geq 0.1$ is normally chosen, and the higher ratio value is 1.

## 4.3 RESULTS

### 4.3.1 EXCEL analysis

A total number of 1036 proteins in normal CSF, previously identified by LCMS, were included in the EXCEL analysis. This analysis showed that only 31.84% of proteins were common to abnormal CSF whilst 68.15% were unique to normal CSF (Figure 4.1).
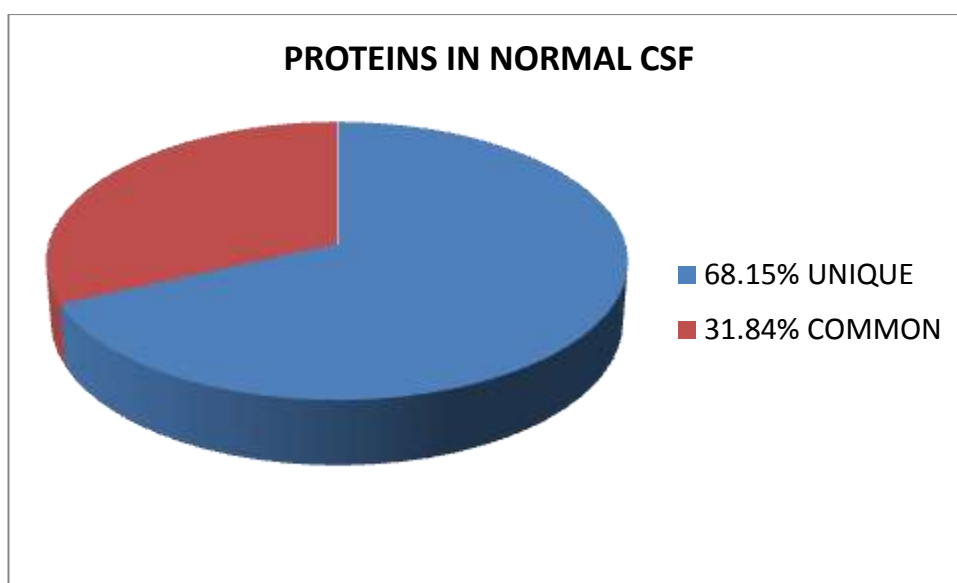


**Figure 4.1. Percentage of proteins presents only in normal CSF versus percentage common to abnormal CSF.**

In contrast, EXCEL analysis demonstrated that the LCMS protein profile from abnormal CSF analysis included a total number of 959 proteins presenting a higher content in proteins common to normal CSF (50.4%) when compared to normal CSF (31.84%). Further, the number of unique proteins to abnormal CSF was smaller with 49.5% of proteins unique to abnormal CSF versus 68.15% found in the normal CSF, (Figure 4.2).
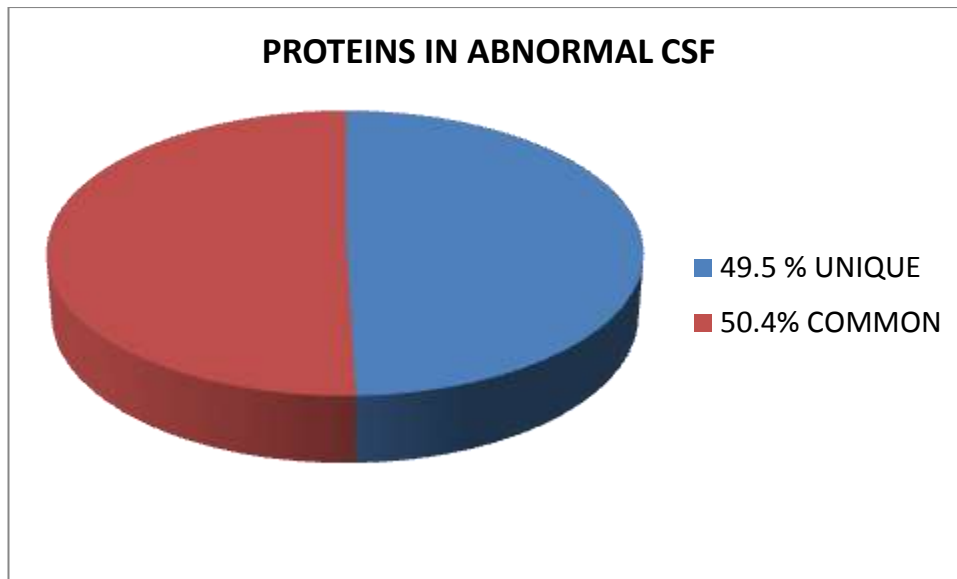
**Figure 4.2. Percentage of protein presents only in abnormal CSF versus percentage common to abnormal CSF.**

Two lists of proteins were produced by EXCEL analysis. They are shown in table 4.1 and table 4.2. Table 4.1 represents proteins found in normal CSF only whereas table 4.2 indicates proteins only found in abnormal CSF. Table 4.1 shows 1036 proteins unique to normal/control CSF out of 1520 total number of proteins analysed. On the other hand, table 4.2 shows how only 475 proteins were unique to abnormal CSF out of 959 total numbers of proteins analysed from abnormal CSF. All tables describe accession numbers (Swisspro database) followed by their chemical names.

TABLE 4.1. **Proteins in normal CSF not present in abnormal CSF**

| PROTEIN ID | CHEMICAL NAME |
|---|---|
| Q9QUT6 | Beta 1 globulin - Rattus sp. |
| Q9QW91 | Beta 2 globulin.- Rattus sp. |
| A25747 | haemoglobin beta III-1/III-2 chain - rat |
| S00840 | Haemoglobin beta chain. minor - rat |
| Q91V15 | Alpha-2-globin chain (2-alpha globin).- Rattus norvegicus - (Rat). |
| Q80XV2 | Alpha-globin (Fragment).- Rattus sp. |
| AAA40756 | RAT Apoliprotein E gene, complete CDS - Rattus norvegicus |
| AAC60703 | S76779 NID: - Rattus sp. |
| B90284 | haemoglobin alpha-2 chain - rat (tentative sequence) (Fragments) |
| BAA01101 | RATBSP NID: - Rattus norvegicus |
| AAF63196 | AF227534 NID: - Rattus norvegicus |
| A36579 | inositol 1,4,5-triphosphate receptor 1 - rat |
| AAF07822 | AF138789 NID: - Rattus norvegicus |
| AAR95660 | AY480027 NID: - Rattus norvegicus |
| AAR95665 | AY480032 NID: -Rattus norvegicus |
| BAA32699 | AB008551 NID: - Rattus norvegicus |
| AAH62238 | BC0662238 NID: - Rattus norvegicus |
| S53611 | MIBP1 protein - rat |
| AAG28596 | AF225960 NID: - Rattus norvegicus |
| A24903 | tubulin alpha-1 chain - Chinese hamster |
| Q923K6 | Calcium channel isoform alpha1E7.- Rattus norvegicus - (Rat). |
| P70570 | Laminin-5 alpha 3 chain.- Rattus norvegicus - (Rat). |
| A60560 | formyltetrahydrofolate dehydrogenase (EC 1.5.1.6)  Rat |

| | |
|---|---|
| AAQ02380 | AY238344 NID: - Rattus norvegicus |
| Q99P38 | Synaptotagmin VIII - Rattus norvegicus - (Rat). |
| Q810E4 | ATP-binding cassette protein C1.- Rattus norvegicus - (Rat). |
| K6PF RAT | 6-phosphofructokinase, muscle type (EC 2.7.1.11) (Phosphofructokinase 1) |
| Q9QYE7 | Alpha D integrin.- Rattus norvegicus - (Rat). |
| KGRTT1 | T-kininogen I precursor- rat |
| A35007 | ATP citrate (pro-S)-lyase (EC 4.1.3.8) - rat |
| Q9JIF2 | Kalirin-9a.- Rattus norvegicus - (Rat). |
| CAE83934 | BX883042 NID: - Rattus norvegicus |
| AAR89949 | AY499658 NID: - Rattus norvegicus |
| Q8CG09 | Multidrug resistance-associated protein 1.- Rattus norvegicus - (Rat). |
| CHRTM1 | Sodium channel protein mu1 alpha chain. skeletal muscle - rat |
| Q811U3 | CAST2.— Rattus norvegicus - (Rat). |
| Q9QUG4 | ATPase 7B.- Rattus norvegicus - (Rat). |
| AAA42014 | RATRBBI NID: - Rattus norvegicus |
| A45386 | omega-conotoxin-sensitive calcium channel alpha-1 subunit rbB-I - rat |
| AAQ96221 | AY383663 NID: - Rattus norvegicus |
| AAS90634 | AY530298 NID: - Rattus norvegicus |
| Q9ERH3 | TGF-beta resistance-associated protein.- Rattus norvegicus - (Rat). |
| Q7TP24 | Bal -667.- Rattus norvegicus - (Rat). |
| AAD41775 | AF121217 NID: - Rattus norvegicus |

| | |
|---|---|
| T32731 | PAR interacting protein - rat |
| Q63581 | Rat T-kininogen (T-KG) - Rattus norvegicus - (Rat). |
| O70611 | Rat skeletal muscle type I voltage-gated sodium channel (rSkM1 ) variant. |
| AAG35188 | AF290214 NID: — Rattus norvegicus |
| BAB78470 | AB049473 NID: - Rattus norvegicus |
| AAQ96270 | AY383712 NID: - Rattus norvegicus |
| AAF61375 | AF133301 NID: - Rattus norvegicus |
| A53165 | dynamin II isoform aa - rat |
| CAA10946 | RNDPDELTA NID: - Rattus norvegicus |
| T17101 | probable voltage-activated cation channel - rat |
| QTP75 | Aa2-066.- Rattus norvegicus - (Rat). |
| AAR23149 | AY462245 NID: - Rattus norvegicus |
| OYRTHX | Heat-stable enterotoxin receptor precursor - rat |
| AAS66223 | AY539883 NID: - Rattus norvegicus |
| AAD04569 | AF102855 NID: - Rattus norvegicus |
| AAO74586 | AY188179 NID: - Rattus norvegicus |
| Q7TPL1 | Ac1-163.- Rattus norvegicus - (Rat). |
| A49128 | cell-fate determining gene Notch2 protein - rat |
| AAF61445 | AF139055 NID: - Rattus norvegicus |
| ATB3 RAT | Plasma membrane calcium-transporting ATPase (EC 3.6.3.8) (PMCA3) |
| AAC68900 | AF086624 NID: - Rattus norvegicus |
| AAB39764 | RATADCYA NID: - Rattus norvegicus |

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|---|---|
| A32827 | fetuin precursor- rat |
| CAE83998 | BX883046 NID: - Rattus norvegicus |
| T42216 | multidrug resistance-associated protein homolog MLP-l - rat |
| ATRTC | actin beta - rat |
| AAC52782 | RNU67309 NID: - Rattus norvegicus |
| AAA41713 | RATNMCA2C NID: - Rattus norvegicus |
| CAE83981 | BX883045 NID: - Rattus norvegicus |
| O54892 | Mutant type II hexokinase. - Rattus norvegicus - (Rat). |
| BAA07283 | RATRCACN4B NID: - Rattus norvegicus |
| BAB61904 | AB053447 NID: - Rattus norvegicus |
| A34736 | nestin - rat |
| WNK4 RAT | Serine/threonine-protein kinase WNK4 (EC 2.7.1.37) |
| AAD42975 | AF159046 NID: - Rattus norvegicus |
| Q99P37 | Synaptotagmin VIIa.- Rattus norvegicus - (Rat). |
| A45669 | neurofilament triplet M protein - rat |
| BAA13016 | D86086 NID: - Rattus norvegicus |
| HHRT60 | chaperonin groEL precursor - rat |
| BAA02499 | RATNMDARC NID: - Rattus norvegicus |
| T140050 | protein kinase (EC 2.7.1.37) beta. myotonic dystrophy-associated - rat |
| CAF33425 | AJ629845 NID: - Rattus norvegicus |
| AAS66670 | AY548105 NID: - Rattus norvegicus |
| AAQ67738 | AY357891 NID: - Rattus norvegicus |
| T17198 | CL3BA protein - rat |

| | |
|---|---|
| Q7TP27 | Bal 651. - Rattus norvegicus |
| SUIS RAT | Sucrase-isomaltase, intestinal Contains: sucrase (EC 3.2.1.48) |
| A345439 | myosin I Heavy chain - rat |
| AAS66285 | AY539945 NID: - Rattus norvegicus |
| A41541 | adenylate cyclase (EC 4.6.1.1) II - rat |
| SYJ1 RAT | Synaptojanin 1 (EC 3.1.3.36) |
| Q9ERQ2 | ABC50 (Fragment).- Rattus norvegicus - (Rat). |
| T43275 | neurabin - rat |
| AAN72415 | AY149995 NID: - Rattus norvegicus |
| AAD16999 | AF110477 NID: - Rattus norvegicus |
| O88421 | Voltage-gated sodium channel variant rPN4a.- Ratlus norvegius - (Rat). |
| O08562 | voltage-dependent sodium channel PN1 (Fragment).- Rattus norvegicus - (Rat). |
| AAS45402 | AY535015 NID: - Rattus norvegicus |
| AAS65873 | AY555273 NID: - Rattus norvegicus |
| CAA44680 | RNFASDNA NID: - Rattus norvegicus |
| CAA31780 | RNFAS NID: - Rattus norvegicus |
| AAQ05020 | AF466694 NID: -Rattus norvegicus |
| Q99P36 | Synaptotagmin VIIb.- Rattus norvegicus - (Rat). |
| A41015 | Dihydrolipoamide S-succinyltransferase (EC 2.3.1.61) precuror - rat |
| T14324 | alpha-latrotoxin receptor, Calcium-independent - rat |
| Q9R1R4 | Tudor repeat associator with PCTAIRE 2.- Rattus norvegicus - (Rat). |

| | |
|---|---|
| AAR16316 | AE017191 NID: - Rattus norvegicus |
| AAA41490 | L29429 NID: - Rattus norvegicus |
| AAN75376 | AY149902 NID: - Rattus norvegicus |
| CAE84039 | BX883048 NID: - Rattus norvegicus |
| T31669 | neural zinc finger protein MyT3 - rat |
| S65440 | Nitric-oxide synthase (EC 1.14.13.39) - rat |
| AAB06393 | RNU59245 NID: - Rattus norvegicus |
| AAC52946 | RNU70050 NID: - Rattus norvegicus |
| AAA74950 | RNU314663 NID: - Rattus norvegicus |
| S46264 | microtubule-associated protein - rat |
| A41939 | G protein-coupled glutamate receptor - rat |
| AAR95632 | AY435131 NID: -Rattus norvegicus |
| Q7TT47 | Paraplegin.- Rattus norvegicus - (Rat). |
| AAR95630 | AY435129 NID: - Rattus norvegicus |
| Q35774 | 75 kDa fibrous sheath protein, - Rattus norvegicus |
| AAO18238 | AY187039 NID: - Rattus norvegicus |
| AAC37646 | RATNR2DA NID: Rattus norvegicus |
| JC4166 | Protein tyrosine kinase (EC 2.7.1.112) tyro3 precursor - rat |
| S46217 | Protein tyrosine-phosphatase (EC 3.1.3.48). Type sigma precursor - rat |
| AAO38858 | AY192567 NID: - Rattus norvegicus |
| Q8CJG5 | DLST dihydrolipoamide succinlytransferase. exon I5 and complete cds.- (Rat). |
| Q9WV40 | Munc13-2 protein.- Rattus norvegicus - (Rat). |

| | |
|---|---|
| Q63557 | Fatty acid Synthase Rattus norvegicus - (Rat). |
| BAA12319 | RATNAP1P NID: - Rattus norvegicus |
| CAA60264 | RNLFIB NID: - Rattus norvegicus |
| A31887 | testosterone 7alpha-monooxygenase (EC 1.4.14.-) cytochrome P450 2A2 - rat |
| AAF62176 | AF247453 NID: - Rattus norvegicus |
| A34337 | propionyl-CoA carboxylase (EC 6.4.1.3) alpha chain precursor - rat (fragment) |
| CAE83972 | BX883045 NID: - Rattus norvegicus |
| Q91ZD9 | Synaptojanin 2B1- Rattus norvegicus - (Rat). |
| AAD24587 | AF136943 NID: - Rattus norvegicus |
| CAA50871 | RNMYR4 NID: - Rattus norvegicus |
| JC2486 | 3',5'-cyclic-nucleotide phosphodiesterase (EC 3.1.4.17). cGMP-stimulated - rat |
| AAL78956 | AF455050 NID: - Rattus norvegicus |
| Q8CIN3 | Receptor-like protein tyrosine phosphatase gamma A-type isoform.-Rat |
| CAB09536 | RNZ96106 NID: - Rattus norvegicus |
| AAF81653 | AF199331 NID: - Rattus norvegicus |
| AAR89918 | AY496443 NID: - Rattus norvegicus |
| T31673 | N-acetylglucosaminyltransferases (EC 2.4.1.-). chain p1 10 - rat |
| BAA06340 | RATACYLCOA NID: - Rattus norvegicus |
| JC5028 | nitric-oxide synthase (EC 1.14.13.39) L - rat |
| Q9JHS0 | Conjugate export pump protein (Fragment).- Rattus norvegicus - |

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| | (Rat). |
| Q8JZS5 | Filamin-interacting protein S-FILIP.- Rattus norvegicus - (Rat). |
| AAP43670 | AY211532 NID: - Rattus norvegicus |
| S18188 | notch protein homolog, - rat |
| AAA18905 | RATRBCII NID: - Rattus norvegicus |
| S58400 | cellutagmin II sytVII - rat |
| I60598 | Fit-1 tyrosine kinase receptor - rat |
| Q8R4C1 | Putative secretory pathway Ca-ATPase SPCA2.- Rattus norvegicus - (Rat). |
| CAC07697 | Sequence 1 from Patent WO9947670.- Rattus norvegicus - (Rat). |
| D3HI RAT | 3-hydroxyisobutyrate dehydrogenase. mitochondrial precursor (EC 1.1.1.31) |
| AAG17548 | AF218826 NID: - Rattus norvegicus |
| AAQ94494 | AY394725 NID: - Rattus norvegicus |
| AAR95631 | AY435130 NID: - Rattus norvegicus |
| AAA40678 | RATADCYB NID: - Rattus norvegicus |
| AAB63955 | RATMATRIN3 NID: - Rattus norvegicus |
| AAA02857 | RATNIIIA NID: - Rattus norvegicus |
| S54357 | plasma membrane Ca2+-ATPase isoform 4 rat |
| BAA28174 | AB001453 NID: - Rattus rattus |
| JS0306 | microtubule-associated protein tau - rat |
| Q8R4D0 | RNA-binding protein staufen splice form A. - Rattus norvegicus - (Rat). |
| AAA02874 | RATGLUR52A NID: -Rattus norvegicus |

| AAD53121 | AF176688 NID: -Rattus norvegicus |
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| S19808 | glutamate receptor GluR5-1 chain precursor - rat |
| A38306 | alpha-mannosidase (EC 3.2.1.24) - rat |
| AAH61793 | BC061793 NID: - Rattus norvegicus |
| AAA41030 | RATCYP45F NID: - Rattus norvegicus |
| Q8K1M7 | BMP retinoic acid-inducible neural-specific protein-3 - Rattus norvegicus |
| BAA07504 | RATF3A NID: - Rattus norvegicus |
| CAA11465 | RNO223599 NID: - Rattus norvegicus |
| BAA88897 | AB020209 NID: - Rattus norvegicus |
| NOS2 RAT | Nitric oxide synthase. inducible (EC 1.14.13.39) (NOS. type II) |
| S37032 | gene LL5 protein - rat |
| AAB37571 | RNU6l266 NID: - Rattus norvegicus |
| Q9R0L5 | TIP120-family protein TIPl20B.- Rattus norvegicus - (Rat). |
| AAP72019 | AY237644 NID: - Rattus norvegicus |
| Q8CF82 | ATP-binding cassette protein 5.- Rattus norvegicus - (Rat). |
| Q63724 | Microtubule-associated protein 2.- Rattus rattus (Black rat). |
| BAA013138 | RATVSMNOS NID: - Rattus norvegicus |
| AAC53338 | AF016297 NID: - Rattus norvegicus |
| AAD30271 | AF127798 NID: - Rattus norvegicus |
| Q8VHE9 | Hypothetical protein.- Rattus norvegicus - (Rat). |
| AAS66226 | AY539886 NID: - Rattus norvegicus |
| AAA84964 | RNU38179 NID: - Rattus norvegicus |
| AAS66282 | AY539942 NID: - Rattus norvegicus |

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| BAA02500 | RATNMDARD1 NID: - Rattus norvegicus |
| S57833 | transmembrane protein HEM2 - rat |
| AAP82454 | AY330294 NID: - Rattus norvegicus |
| CAE83957 | BX883044 NID: - Rattus norvegicus |
| AAR95633 | AY435132 NID: - Rattus norvegicus |
| CGRT1S | collagen alpha 1(I) chain - rat (tentative sequence) (fragments) |
| CA11 RAT | Collagen alpha 1(I) chain (Fragments) -Rattus norvegicus |
| AAD42976 | AF159047 NID: - Rattus norvegicus |
| T14124 | neural zinc finger factor 3 - rat |
| BAD15356 | AB176831 NID: - Rattus norvegicus |
| AAF81646 | AF199324 NID: - Rattus norvegicus |
| AAA70429 | RAT10HCO NID: Rattus norvegicus |
| PIB4 RAT | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 4 |
| AAS18426 | AY495696 NID: - Rattus norvegicus |
| A38235 | microtubule-associated protein. 110K tau - rat |
| Q55166 | ARE1-Rattus norvegicus |
| Q4RTV3 | vitamin D3 25-monooxygenase (EC 1.14.14.-) cytochrome P450 27 precursor. |
| Q7TPJ1 | Ac2-233 - Rattus norvegicus - (Rat). |
| AAQ16157 | AY273816 NID: -Rattus norvegicus |
| P97949 | TAP1 protein.- Rattus norvegicus - (Rat). |
| A40170 | glutamate receptor A precursor- rat |
| Q63555 | SP120.- Rattus norvegicus (Rat). |
| BAA07994 | RATNWUT NID: - Rattus norvegicus |

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| AAA41096 | RATDPP NID: - Rattus norvegicus |
| Q9JMK8 | Vps541-like protein.- Rattus norvegicus - (Rat) |
| A34272 | testosterone 7alpha-hydroxylase (EC 1.14.14.-) cytochrome P4502A1 - rat |
| Q7TP38 | Ab2-371 - Rattus norvegicus - (Rat). |
| JC5029 | nitric-oxide synthase (EC 1.14.13.39) U - rat |
| JH0592 | glutamate receptor chain KA-2 precursor - rat |
| CAA56333 | RNHEM2 NID: - Rattus sp. |
| AAS18425 | AY495695 NID: - Rattus norvegicus |
| I56555 | Sodium channel protein 6 - rat |
| A37180 | chromogranin/secretogranin-like vesicle protein precursor - rat |
| JC7365 | septin-like protein-a - rat |
| AAD46653 | AF164486 NID: - Rattus norvegicus |
| O88600 | Ischemia responsive 94 kDa protein - Rattus norvegicus - (Rat). |
| A53588 | adenylate cyclase (EC 4.6.1.1) VIII - rat |
| O54728 | Phospholipase B.- Rattus norvegicus - (Rat). |
| AAB60525 | U45479 NID: - Rattus norvegicus |
| AAA40813 | RATBG NID: - Rattus norvegicus |
| I57961 | glucuronosyltransferase (EC 2.4.1.17) precursor - rat |
| A54142 | nucleoporin NUP107 - rat |
| CAD89523 | RNO557016 NID: - Rattus norvegicus |
| AAH61999 | BC061999 NID: - Rattus norvegicus |
| A39914 | dipeptidyl-peptidase IV (EC 3.4.14.5). membrane-bound form precursor - rat |

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| OYRTR | atrial natriuretic peptide receptor A precursor - rat |
| Q9ERZ8 | Vanilloid receptor-related osmotically activated channel.- Rattus norvegicus |
| AAD09246 | RNU90725 NID: - Rattus norvegicus |
| Q63643 | Aryl hydrocarbon reccptor nuclear translocator 2 (Fragment).- Rat |
| AAC13318 | RNU61772 NID: - Rattus norvegicus |
| AAQ96246 | AY383688 NID: - Rattus norvegicus |
| AAC52693 | MMU58203 NID: -Mus musculus |
| AAS66255 | AY539915 NID: - Rattus norvegicus |
| O54755 | MIPP65 - Rattus norvegicus - (Rat). |
| AAA17833 | RNU08260 NID: - Rattus norvegicus |
| AAD50906 | PAIRED 1G-LIKE RECEPTOR-A2.- Rattus norvegicus - (Rat). |
| AAO91938 | AY191271 NID: - Rattus norvegicus |
| JC4391 | pyruvate carboxylase (EC 6.4.1.1) precursor - rat |
| AAF25004 | AF156982 NID: - Rattus norvegicus |
| Q9QW24 | TIE-2 =RECEPTOR-like tyrosine kinase - Rattus sp. |
| Q9EPI7 | Putative MAGE-like protein.- Rattus norvegicus |
| AAQ88212 | AY382879 NID: - Rattus norvegicus |
| AAH59165 | BC059165 NID: - Rattus norvegicus |
| AAH61865 | BC061865 NID: - Rattus norvegicus |
| CAE83974 | BX883045 NID: - Rattus norvegicus |
| AAA40844 | RATCACAI NID: - Rattus norvegicus |
| BAC36835 | AK077507 NID: - Mus musculus |
| S70642 | ubiquitin ligase Nedd4 - rat (fragment) |

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| Q7TSP2 | Kinesin-like protein KIF15.- Rattus norvegicus - (Rat). |
| JC6124 | diacylglycerol kinase (EC 2.7.1.107) IV - rat |
| AAB96342 | RNU82626 NID: - Rattus norvegicus |
| S68443 | double-stranded RNA-specific adenosine deaminase (EC 3.5.4.-) - rat |
| Q923K9 | APOBEC-1 complementation factor long isoform.- Rattus norvegicus - (Rat). |
| AAA41240 | RATGLUR2A NID: - Rattus norvegicus |
| AAD34012 | AF149118 NID: - Rattus norvegicus |
| PLD1 RAT | Phospholipase D1 (EC 3.1.4.4) (PLD 1) (Choline phosphatase 1) |
| T13725 | phospholipase D (EC 3.1.4.4) 1a - rat |
| A24600 | glucuronosyltransferase (EC 2.4.1.17) precursor - 3-methylcholanthrene-inducible |
| Q63283 | LMW T-kininogen I.- Rattus norvegicus - (Rat). |
| A57112 | contactin precursor - rat |
| T17199 | CL3BB protein - rat |
| CAB44314 | RNO131899 NID: - Rattus norvegicus |
| AAB96356 | RNU40603 NID: - Rattus norvegicus |
| Q8VD43 | UDP-glucuronosyltransferase 1A7.- Rattus norvegicus - (Rat). |
| AAC33405 | RNU95177 NID: - Rattus norvegicus |
| P97561 | Tap1 protein.- Rattus norvegicus - (Rat). |
| Q925T1 | RGPR-p1I7.- Rattus norvegicus - (Rat). |
| AAH61722 | BC061722 NID: - Rattus norvegicus |
| CAA09720 | RNO011605 NID: - Rattus norvegicus |

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| AAC33291 | AF083330 NID: - Rattus norvegicus |
| Q7TQP6 | Cardiac ankyrin repeat kinase.- Rattus norvegicus - (Rat). |
| S13426 | multidrug resistance protein homolog - rat |
| AAH61738 | BC061738 NID: - Rattus norvegicus |
| AAF81655 | AF199333 NID: - Rattus norvegicus |
| O88763 | phosphatidylinositol 3-kinase Rattus norvegicus - (Rat). |
| NAL6 RAT | NACHT-. LRR- and PYD-containing protein 6 |
| Q9R174 | Glutamate receptor subunit GluR2-flip precursor - Rattus norvegicus - (Rat). |
| B28065 | Ca2+ transporting ATPase (EC 3.6.3.8) 2 - rat |
| GEPH RAT | Gephyrin (Putative glycine receptor-tubulin linker protein).- Rat |
| Q91XQ4 | Putative ionotropic glutamate receptor GLURR-F11694B.- Rat |
| Q9JE0 | MHC class II transactivator type I.- Rattus norvegicus - (Rat). |
| BAA12144 | D83948 NID: - Rattus norvegicus |
| A44097 | methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27) - rat |
| AAA02853 | RATNIIIA NID: - Rattus norvegicus |
| A29953 | alpha-I proteinase inhibitor III. hepatic clone AF7 - rat (fragment) |
| AAF81644 | AF199322NID: - Rattus norvegicuss |
| T00025 | PSD-95 binding protein - rat |
| Q9R0T3 | Protein kinase Inhibitor p58.- Rattus norvegicus (Rat). |
| JC6329 | yeast secretory protein homolog rsec6/8 complex - rat |
| BAA09460 | RATTESK1B NID: - Rattus norvegicus |
| BAA23672 | D89093 NID: - Rattus norvegicus |

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| AAD16009 | AF120492 NID: - Rattus norvegieus |
| Q9JL97 | GPI-anchored ceruloplasmin.- Rattus norvegicus - (Rat). |
| A39667 | brain-derived neurotrophic factor receptor precursor - rat |
| Q8VD44 | UDP-glucurunosyltransferase 1A8.- Rattus norvegicus - (Rat). |
| S34719 | haemoglobin beta-2.0 chain - rat |
| AAH58502 | BC058502 NID: - Rattus norvegicus |
| JC5953 | inter-alpha-inhibitor HP4 heavy chain - rat |
| AAS21244 | AY462092 NID: - Rattus norvegicus |
| AAR01113 | AY333961 NID: - Rattus norvegicus |
| AAK56958 | AF375463 NID: - Rattus norvegicus |
| S41067 | collagen alpha 1(III) chain - rat |
| Q9R0W3 | Plasminogen protein precursor (EC 3.4.21.7).- Rattus norvegicus - (Rat). |
| AAS66221 | AY539881 NID: - Rattus norvegicus |
| AAR95634 | AY435133 NID: - Rattus norvegicus |
| A53731 | translation initiation factor eIF-2 alpha chain kinase (EC 2.7.1.-) - rat |
| CAA90600 | RNPTPASE NID: - Rattus norvegicus |
| BAA23523 | AB008803 NID: - Rattus norvegicus |
| BAA75254 | AB011821 NID: - Rattus norvegicus |
| S01713 | tubulin beta-7 chain - chicken |
| A35781 | hippocampus-derived neurotrophic factor precursor - rat |
| Q924K3 | APOBEC-1 complementation factor short isoform.- Rattus norvegicus - (Rat). |

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| AAB09537 | RNU63288 NID: - Rattus norvegicus |
| Q35850 | Carboxypeptidase D precursor- Rattus norvegicus - (Rat). |
| Q9JIZ3 | Cenexin 2.- Rattus norvegicus - (Rat). |
| Q62965 | Glycine-, glutamate-, thienylcyclohexylpiperidine-binding protein.- Rat |
| PC6300 | synaptotagmin X - rat (fragment) |
| Q9R0L3 | TIP120-family protein TIP120B, short form.- Rattus norvegicus - (Rat). |
| P97560 | Tap1 protein.- Rattus norvegicus - (Rat). |
| Q8K3M6 | Cytomatrix protein p110 - Rattus norvegicus - (Rat). |
| Q7TP87 | AB1-233.- Rattus norvegicus - (Rat). |
| S25576 | probable transport protein mt2 - rat |
| AAC52289 | RNU24150 NlD: - Rattus norvegicus |
| AAA63911 | RATOSTP NID: - Rattus norvegicus |
| AAF34909 | AF221622 NID: - Rattus norvegicus |
| Q80WI4 | Phospholipase C delta.- Rattus sp. |
| CAA67021 | RNKISRNA NID: - Rattus norvegicus |
| BAD05181 | AB118026 NID: - Rattus norvegicus |
| CAB56206 | RNO245646 NID: - Rattus norvegicus |
| I77467 | serotonin receptor ID - rat |
| Q811A4 | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2. long variant.- Rat |
| AAC52207 | RNU09357 N1D: - Rattus norvegicus |
| AAQ02379 | AY238343 NID: - Rattus norvegicus |

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| B55915 | guanylate cyclase (EC 4.6.1.2) 2F precursor - rat |
| AAA17831 | RNU08258 NID: - Rattus norvegicus |
| BAD06369 | AB113387 NID: - Rattus norvegicus |
| Q923K1 | Sweet taste receptor T1R3.- Rattus norvegicus - (Rat). |
| Q62819 | Aromatic L-amino acid decarboxylase - Rattus norvegicus (Rat). |
| AAA73342 | RATALTSPLB NID: - Rattus norvegicus |
| AAA85463 | RATVDCCA NID: - Rattus norvegicus |
| Q9WUF7 | Hyaluronan receptor RHAMM.- Rattus norvegicus - (Rat). |
| Q9JJS5 | SH3-domain binding protein 4.- Rattus norvegicus - (Rat). |
| AAQ23131 | AY345164 NID: - Rattus norvegicus |
| BAD08353 | AB116149 NID: - Rattus norvegicus |
| I56577 | 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (EC 3.1.4.37) - rat |
| JH0422 | voltage-dependent calcium channel complex. alpha-1 chain - rat |
| Q9ER34 | Mitochondrial aconitase precursor.- Rattus norvegicus - (Rat). |
| AAQ96263 | AY383705 NID: - Rattus norvegicus |
| AAA41080 | RATDBP NID: - Rattus norvegicus |
| CAD48615 | Sequence 11 from Patent WO2059612.- Rattus norvegicus - (Rat). |
| BAD01656 | AB096213 NID: - Rattus norvegicus |
| CAA78936 | RNGLURCA NID: - Rattus norvegicus |
| XORTDH | Xanthine dehydrogenase (EC 1.1.1.204) / Xanthine oxidase (EC 1.1.3.22) - rat |
| AAS66290 | AY539950 NID: - Rattus norvegicus |
| TAU RAT | Microtubule-associated protein tau (Neurofibrillary tangle protein) |
| B28821 | 1-phosphatidylinositol-4.5-bisphosphate phosphodiesterase (EC |

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| | 3.1.4.11) delta-1 |
| CAA27067 | RNTUBB15 NID: - Rattus norvegicus |
| Q9Z1X1 | GLUT4 vesicle protein.- Rattus norvegicus - (Rat). |
| CAA49905 | RNENACA NID: - Rattus norvegicus |
| MOES RAT | Moesin (Membrane-organizing extension spike protein).- Rat |
| AJRTRS | argininosuccinate synthase (EC 6.3.4.5) - rat |
| I59422 | rsec8 - rat (fragment) |
| A25113 | tubulin beta chain 15- rat |
| AAM75358 | AF521008 NID: - Rattus norvegicus |
| P70625 | Zonula occludens 2 protein (Fragment).- Rattus norvegicus - (Rat). |
| T42842 | bile salt transport protein. ATP-dependent - rat |
| AAG29823 | AF304446 NID: - Rattus norvegicus |
| AAA41280 | RATGRT NID: - Rattus norvegicus |
| D40170 | glutamate receptor - rat |
| CAA07267 | RNAJ6855 NID: - Rattus norvegicus |
| OXRTA1 | acyl-CoA oxidase (EC 1.3.3.6) chain A. peroxisomal splice form 1- rat |
| Q9QXU8 | Dynein light intermediate chain 1.- Rattus norvegicus - (Rat). |
| A30363 | glycoprotein GP330. renal - rat (fragments) |
| AAC36065 | AF057026 NID: - Rattus norvegicus |
| AAA96256 | RNU32314 NID: - Rattus norvegicus |
| S55255 | lamina-associated protein 2 - rat |
| AAN59930 | AF548738 NID: - Rattus norvegicus |
| AAB47753 | RNU81035 NID: - Rattus norvegicus |

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| UD18 RAT | UDP-glucuronosyltransferase 1-8 precursor. microsomal |
| AAG23699 | AF311311 NID: - Rattus norvegicus |
| BAD01114 | AB119249 NID: - Rattus norvegicus |
| AAC33292 | AF083331 NID: - Rattus norvegicus |
| Q9ESH7 | Kinesin light Chain KLCt.- Rattus norvegicus - (Rat). |
| A48758 | protein tyrosine-phosphatase (EC 3.1.3.48). receptor-linked form Pl precursor |
| CAA67142 | RNMME NID: -Rattus norvegicus |
| CAC08185 | RNO293948 NID: - Rattus norvegicus |
| Q7TP84 | Ab1 -346 - Rattus norvegicus (Rat ). |
| AAR20710 | AY455861 NID:Rattus norvegicus |
| CAA35050 | RNGLUR1 NID: - Rattus norvegicus |
| AAQ91054 | AY387084 NID: - Rattus norvegicus |
| S15904 | alpha-l proteinase inhibitor III. variant 1 precursor - rat |
| AAB06043 | RNU58857 NID: - Rattus norvegicus |
| AAD10403 | RNU57836 NID: - Rattus norvegicus |
| XNTRO | ornithine-oxo-acid transaminase (EC 2.6.1.13) precursor - rat |
| Q9QW67 | LAR. leukocyte common antigen-related |
| Q8CIZ0 | ERC2.- Rattus norvegicus - (Rat). |
| S19595 | chloride channel protein - rat |
| A33505 | somatotropin receptor precursor - rat |
| B48758 | protein-tyrosine-phosphatase (EC 3.1.3.48). receptor-linked form PS precursor - |
| AAS66224 | AY539884 NID: - Rattus norvegicus |

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| JH0790 | lipoprotein lipase (EC 3.1.1.34) precursor - rat |
| AAF74258 | AF227741 NID: - Rattus norvegicus |
| S08211 | dnaK-type molecular chaperone hst70 - rat |
| Q8VH46 | Actin filament associated protein.- Rattus norvegicus - (Rat). |
| AAL84229 | AF446001 NID: - Rattus norvegicus |
| T10811 | channel associated protein of synapse 2 - rat |
| A39368 | sterol carrier protein 2 precursor - rat |
| S07624 | acylaminoacyl-peptidase (EC 3.4.19.1) - rat |
| BAA83593 | AB022699 NID: - Rattus norvegicus |
| Q8R458 | Protein kinase for Splicing component.- Rattus norvegicus - (Rat). |
| BAB21439 | AB046442 NID: - Rattus norvegicus |
| AAP92621 | AY325220 NID: - Rattus norvegicus |
| AAC33895 | AF068202 NID: - Rattus norvegicus |
| AAA40608 | RAT3H3M NID: -Rattus norvegicus |
| AAL27988 | AF350422 NID: - Rattus norvegicus |
| JH0811 | acetylcholinesterase (EC 3.1.1.7) catalytic chain precursor - rat |
| AAH34645 | B034645 NID: - Mus musculus |
| T46635 | phospholipase D (EC 3.1.4.4) 1a - rat |
| AAA42284 | RATTRKCC NID: - Rattus norvegicus |
| O08721 | transmembrane receptor UNC5H1.- Rattus norvegicus - (Rat). |
| Q9JMG8 | Dihydropyrimidinase-related protein.- Rattus norvegicus - (Rat). |
| BAC97816 | AB100356 NID: - Rattus norvegicus |
| AAS78929 | AY533525 NID: - Rattus norvegicus |
| O88752 | Epsilon 1 globin.- Rattus norvegicus - (Rat). |

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| BAA13413 | D87515 NID: - Rattus norvegicus |
| Q9R1L1 | NBC-like protein 3.- Rattus norvegicus |
| AAA41625 | RATMLCK NID: -Rattus norvegicus |
| BAA09091 | RATCLC5 NID: - Rattus norvegicus |
| AAB71745 | RNU76206 NID: -Rattus norvegicus |
| CAE83966 | BX883044 NID: - Rattus norvegicus |
| AAB48046 | RNU88294 NID: - Rattus norvegicus |
| BAB23530 | AK004749 NID: - Mus musculus |
| Q8CG07 | Werner synodrome-interacting protein-like protein. - Rattus norvegicus - (Rat). |
| RIN1 RAT | Ras and Rab interactor 1 (Rat interaction/interference protein 1).- Rat |
| AAB58256 | RNU80076 NID: - Rattus norvegicus |
| AAS66220 | AY539880 NID: - Rattus norvegicus |
| BAD13422 | AB126168 NID: - Rattus norvegicus |
| BAB19011 | AB052294 NID: - Rattus norvegicus |
| Q80Z07 | ATP-binding cassette protein 5.- Rattus norvegicus - (Rat). |
| AAL73196 | AF335505 NID: - Rattus norvegicus |
| CAC21554 | RNO298278 NID: - Rattus norvegicus |
| SI3064 | 1D-myo-inositol-trisphosphate 3-kinase (EC 2.7.1.127) A - rat |
| AAD52710 | AF121893 NID: - Rattus norvegicus |
| AAN64275 | AY145899 NID: - Rattus norvegicus |
| BAA90553 | AB032551 NID: - Rattus norvegicus |
| O70408 | Outer dense fiber protein ODF84.- Rattus norvegicus - (Rat). |

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| AAA41392 | RATIGFIRT NID: - Rattus norvegicus |
| AAL84222 | AF445994 NID: - Rattus norvegicus |
| AAB86910 | RNU69550 NID: - Rattus norvegicus |
| AAP82452 | AY330292 NID: - Rattus norvegicus |
| MLRS RAT | Myosin regulatory light chain 2, skeletal muscle isoform |
| Q811M5 | Complement factor 6.- Rattus norvegicus - (Rat). |
| AAC52849 | RNU55836 NID: - Rattus norvegicus |
| A44839 | glutamate receptor 4c - rat |
| AAH67245 | BC067245 NID: - Rattus norvegicus |
| AAA83235 | RNU40652 NID: - Rattus norvegicus |
| RIP1 RAT | Peripheral-type benzodiazepine receptor-associated protein 1 |
| Q9JLS3 | Serine/threonine protein kinase TAO2.- Rattus norvegicus - (Rat). |
| A35956 | thyrotropin receptor - rat |
| A42497 | anion exchanger 3, cardiac splice form - rat |
| AAP42419 | AY208851 NID: - Rattus norvegicus |
| T08423 | Axin homolog Axil - rat |
| CAE83959 | BX883044 NID: - Rattus norvegicus |
| OXRTA2 | acyl-CoA oxidase (EC 1.3.3.6) chain A, peroxisomal Splice form II rat |
| S02003 | neurofilament triplet H protein - rat (fragment) |
| S40482 | serine/threonine-specific protein kinase (EC 2.7.1.-) - rat |
| AAB03365 | RNU08136 NID: - Rattus norvegicus |
| AAD25916 | AF072509 NID: - Rattus norvegicus |
| AAF05769 | AF194371 NID: - Rattus norvegicus |

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| AAA88511 | RATP4502A3 NID: -Rattus norvegicus |
| AAD22522 | AF091457 NID: - Rattus norvegicus |
| AAA41457 | RATITK NID: - Rattus norvegicus |
| AAF70456 | AF221952 NID: - Rattus norvegicus |
| Q811X6 | CRY - Rattus norvegicus - (Rat). |
| A48801 | 2-hydroxyacylsphingosine 1-beta-galactosyltransferase (EC 2.4.1.45) precursor |
| O54858 | Carboxypeptidase Z.- Rattus norvegicus - (Rat). |
| BAD11366 | AB162856 NID: - Rattus norvegicus |
| A34307 | Ca2+ transporting ATPase (EC 3.6.3.8) - rat |
| Q7TMR7 | Monocarhoxylate transporter 7B splice variant.- Rattus norvegicus - (Rat). |
| Q91XJ3 | IIIG9 long form.- Rattus norvegicus - (Rat). |
| AAC19367 | AF067793 NID: - Rattus norvegicus |
| CAA39884 | RNNG2 NID: - Rattus norvegicus |
| JC2447 | carboxylesterase (EC 3.1.1.1) ES-3 precursor - rat |
| AAB05930 | RNU63971 NID: - Rattus norvegicus |
| S28857 | glutamate receptor delta-1 chain precursor- rat |
| AAP82451 | AY330291 NID: - Rattus norvegicus |
| P97559 | Tap1 protein.- Rattus norvegicus - (Rat). |
| PN0473 | valine-tRNA ligase (EC 6.1.1.9) - rat (fragment) |
| CAD54073 | RNO511273 NID: - Rattus norvegicus |
| Q8K1M8 | BMP/retinoic acid-inducible neurai-specific protein-2 - Rattus norvegicus |

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| Q9Z2J3 | S-nexilin.- Rattus norvegicus - (Rat). |
| Q9Z2J4 | F-actin binding protein b-Nexilin.- Rattus norvegicus - (Rat). |
| BAA25987 | D82883 NID: - Rattus norvegicus |
| AAB24228 | S48813 NID: - Rattus sp. |
| A42764 | Ca2+ transporting ATPase (EC 3.6.3.8) - rat |
| JH0527 | carbonate dehydratase (EC 4.2.1.1) II - rat |
| CAA15426 | RNO236911 NID: - Rattus norvegicus |
| A26186 | pyruvate kinase (EC 2.7.1.40) isozyme M2 - rat |
| Q9QXF7 | CYP2J4 protein.- Rattus norvegicus - (Rat). |
| Q7TSB4 | ADAM28 isoform-1.- Rattus norvegicus - (Rat). |
| Q8R427 | ATP-binding cassette protein Blb.- Rattus norvegicus - (Rat). |
| O35265 | Putative pheromone receptor.- Rattus norvegicus - (Rat). |
| T13957 | period protein PER2 - rat |
| BAA06170 | RATP130CAS NID: - Rattus norvegicus |
| Q63618 | Espin.- Rattus norvegicus - (Rat). |
| TVRTNU | protein-tyrosine kinase (EC 2.7.1.112) neu precursor - rat |
| Q7TNA8 | Lactate dehydrogenase A-like protein.- Rattus norvegicus - (Rat). |
| A34164 | Cholestrol monooxygenase (side-chain-cleaving) |
| Q7TT74 | Chloride channel-isoform 5 (Fragment).- Rattus norvegicus (Rat) |
| ABG8 RAT | ATP-binding cassette, sub-family G. member 8 (Sterolin - 2) Rat |
| O88370 | Phosphatidylinositol 5-phosphate 4-kinase gamma.- Rattus norvegicus - (Rat). |
| AAH60586 | BC060586 NID: - Rattus norvegicus |
| AAB49519 | S83279 NID: - Rattus sp. |

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| Q920D5 | Capsase-12 - Rattus norvegicus - (Rat). |
| AAC23707 | RNU91561 NID: - Rattus norvegicus |
| BAA94745 | AB041723 NID: - Rattus norvegicus |
| BAA14107 | D90059S5 NID: - Rattus norvegicus |
| AAQ91044 | AY387074 NID: - Rattus norvegicus |
| AAQ96248 | AY383690 NID: - Rattus norvegicus |
| S27258 | activin receptor type II - rat |
| Q9JKW1 | TIM 22 preprotein translocase (Fragment).- Rattus norvegicus - (Rat). |
| S53531 | gamma-aminobutyric acid beta 2 chain - mouse |
| BAD06375 | AB113393 NID: - Rattus norvegicus |
| AAS78661 | AY496961 NID: - Rattus norvegicus |
| Q91WX9 | Voltage-gated calcium channel pore forming subunit CaV1. 3 alpha I - Rat |
| Q80W87 | ROBO4.- Rattus norvegicus - (Rat). |
| S29509 | insulysin (EC 3.4.24.56) precursor [validated] - rat |
| A27366 | AMP deaminase (EC 3.5.4.6), skeletal muscle - rat |
| AAG37066 | AF306546 NID: - Rattus norvegicus |
| C40170 | glutamate receptor C precursor - rat |
| AAB03281 | RNUS2103 NID: - Rattus norvegicus |
| A61208 | chondroitin sulfate proteoglycan NG2 - rat |
| Q8CFC4 | Histo-blood group ABO transferase (B blood group galactosyltransferase) |
| I56333 | apolipoprotein B - rat (fragment) |

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| O55007 | Tulip 1.- Rattus norvegicus - (Rat). |
| BAA23122 | AB008422 NID: - Rattus norvegicus |
| I58159 | protein kinase C-regulated chloride channel - black rat |
| AAB51398 | RNU93052 NID: - Rattus norvegicus |
| A46748 | Na+/H+ exchanging protein NHE-2 - rat |
| OYRTA1 | guanylate cyclase (EC 4.6.1.2). soluble, alpha-1 chain - rat |
| CAA10046 | RNO012482 NID: - Rattus norvegicus |
| Q9EQM9 | Angiotensin-converting enzyme.- Rattus norvegicus - (Rat). |
| Q7TQ85 | Ac1 164- Rattus norvegicus - (Rat). |
| CAA34160 | RNALPH03 NID: - Rattus norvegicus |
| Q8R3Z7 | Pincher.- Rattus norvegicus - (Rat). |
| 1BG3A | hexokinase (EC 2.7.1.1), chain A - norway rat (fragments) |
| 1BG3B | hexokinase (EC 2.7.1.1), chain B - norway rat (fragments) |
| NFAS RAT | Neurofascin precursor.- Rattus norvegicus - (Rat). |
| AAA41672 | RATNALPH2 NID: - Rattus norvegicus |
| Q9JMA8 | Reg receptor.- Rattus norvegicus - (Rat). |
| I65413 | sodium-dependent neurotransmitter transporter - rat (fragment) |
| CAC08993 | Sequence 1 from Patent WO0026348.- Rattus norvegicus |
| Q925T8 | BMP/retinoic acid-inducible neural-specific protein.- Rattus |
| HCN2 RAT | Potassium/sodium yperpolarization-activated cyclic nucleotide-gated channel |
| O35470 | cAM P-specific phosphodiesterase.- Rattus norvegicus - (Rat). |
| Q9Z221 | Outer dense fiber ODF3.- Rattus norvegicus - (Rat). |
| BAC21666 | AB073215 NID: - Rattus norvegicus |

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| A42046 | surfactant protein D - rat |
| BAA09922 | RATPBMP3 NID: - Rattus norvegicus |
| Q80XF7 | Gap junction channel protein conncxin47 (Fragment).- Rattus norvegicus - (Rat). |
| AAQ96245 | AY383687 NID: - Rattus norvegicus |
| CAD13342 | RNO421447 NID: - Rattus norvegicus |
| Q8CFG7 | Calcium channel alpha-2 delta -1 subunit isoform e.- Rattus norvegicus - (Rat). |
| A44147 | calcium channel protein alpha-2 chain precursor - rat |
| BAA22224 | D85509 NID: - Rattus rattus |
| Q9ER28 | Endothelial type gp91-phox.- Rattus norvegicus - (Rat). |
| A36121 | aromatase (EC 1.14.14.-) cytochrome P450 19 - rat |
| JC6136 | kexin-like protein convertase (EC 3.4.-.-) - rat |
| Q9R1Q3 | Glial fibrillary acidic protein alpha.- Rattus norvegicus - (Rat). |
| AAA41476 | RATKEX2A NID: - Rattus norvegicus |
| BAD06378 | ABII3396 NID: - Rattus norvegicus |
| Q9QYJ5 | PDE10A3 (EC 3.1.4.17) Rattus norvegicus - (Rat). |
| AAA74248 | RATC1H4SY NID: - Rattus norvegicus |
| AAA40674 | RATACTRII NID: - Rattus norvegicus |
| AAL84224 | AF445996 NID: - Rattus norvegicus |
| AAQ96256 | AY383698 NID: - Rattus norvegicus |
| EGRT | epidermal growth factor precursor - rat |
| TRIF RAT | Troponin I, fast skeletal muscle (Troponin I, fast-twitch isoform) - Rat |

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| Q8VHN2 | NMDA ligand-gated receptor 3B subunit.- Rattus norvegicus |
| Q9Z213 | Hypothetical protein.- Rattus norvegicus - (Rat). |
| O55163 | Proton-gated cation channels modulatory subunit MDEG2.- Rattus norvegicus |
| AAB96560 | RNU95748 NID: - Rattus norvegicus |
| AAA56637 | RNSGIII NID: - Rattus norvegicus |
| AAG23357 | Beta ig-h3 (Fragment).- Rattus norvegicus - (Rat). |
| AAQ96259 | AY383701 NID: - Rattus norvegicus |
| AAH63161 | BC063161 NID: - Rattus norvegicus |
| B34488 | calpain (EC 3.4.22.17) large chain 3 - rat |
| AAA41043 | RATCYPA NID: - Rattus norvegicus |
| CAA62002 | RNTSP4 NID: - Rattus norvegicus |
| Q7TP21 | Cb1-727 - Rattus norvegicus - (Rat). |
| Q06884 | Testosterone-6-beta hydroxylase.- Rattus norvegicus - (Rat). |
| C43274 | N-methyl D-aspartate receptor (NMDR) |
| P70540 | Peroxisomal multifunctional enzyme type II.- Rattus norvegicus |
| Q9JJL4 | GTP-binding protein tc10.- Rattus norvegicus (Rat ). |
| S00289 | alkaline phosphatase (EC 3.1.3.1), hepatic precursor - rat |
| Q9JM01 | Serine/threonine kinase NKIATRE alpha.- Rattus norvegicus |
| AAA99237 | RATSUR NID: - Rattus norvegicus |
| AAG28597 | AF225961 NID: - Rattus norvegicus |
| Q80ZE7 | CD2AP/CMS.- Rattus norvegicus - (Rat). |
| A28166 | Kupffer cell receptor - rat |
| CAC13969 | RNO250313 NID: - Rattus norvegicus |

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|---|---|
| KHRTH | cathepsin H (EC 3.4.22.16) precursor - rat |
| CAC13957 | RNO250329 NID: - Rattus norvegicus |
| SYRTDT | aspartate-tRNA ligase (EC 6.1.1.12) - rat |
| Q9QX10 | Na-K-Cl cotransporter.- Rattus norvegicus - (Rat). |
| JC4239 | phospholipase A2-activating protein - rat |
| Q9EPI1 | Xylosyltransferase I (EC 2.4.2.26) (Fragment).- Rattus norvegicus |
| KCB2 RAT | Potassium voltage-gated channel subfamily B member 2 |
| P70539 | Activin receptor-like kinase 7.- Rattus norvegicus - (Rat). |
| CAB61692 | RNO251245 NID: - Rattus norvegicus |
| AAC39689 | AF004563 NID: - Homo sapiens |
| Q9Z1Z9 | LIM-domain protein LMP-1.- Rattus norvegicus - (Rat). |
| Q810C2 | Spergen-2.- Rattus norvegicus - (Rat). |
| Q9ER23 | RP59 protein.- Rattus norvegicus - (Rat). |
| CAF25053 | AJ626743 NID: - Rattus norvegicus |
| AAF19354 | AF185637 NID: - Rattus norvegicus |
| DIRTR1 | protein-arginine deiminase (EC 3.5.3.15) 1 - rat |
| R5RTL7 | ribosomal protein L7. cytosolic [validated] - rat |
| Q7TN97 | Keratin 5.- Rattus norvegicus - (Rat). |
| AAH62060 | BC062060 NID: - Rattus norvegicus |
| S60588 | drebrin A - rat |
| Q9QYV3 | C3G protein (Fragment).- Rattus norvegicus - (Rat). |
| A28114 | alkaline phosphatase (EC 3.1.3.1) precursor - rat |
| A25516 | propionyl-CoA carboxylase (EC 6.4.1.3) beta chain precursor - rat |
| Q9JMA0 | tRNA-guanine transglycosylase (EC 2.4.2.29). - (Rat). |

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| Q63662 | Rat 3-methylcholanthrene-inducible truncated UDP glucuronosyltransfrase |
| AAR92226 | AY389807 NID: - Rattus norvegicus |
| AAA41065 | RATCYPPB NID: - Rattus norvegicus |
| CAA00863 | BRL-3A BINDING PROTEIN PRECURSOR.- Rattus norvegicus - (Rat). |
| AAC52855 | RNU59241 NID: - Rattus norvegicus |
| A36443 | seminal vesicle secretory protein II precursor - rat |
| AAD27869 | AF133731 NID: - Rattus norvegicus |
| ENOB RAT | Beta enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) |
| Q91YB6 | Complement inhibitory factor H.- Rattus norvegicus |
| CAA58233 | RNPAIHC3 NID: - Rattus norvegicus |
| P70603 | Activin-like receptor kinase-7 - Rattus norvegicus |
| CAE84034 | BX883048 NID: - Rattus norvegicus |
| A53455 | syntaxin-binding protein n-Secl - rat |
| O54720 | Kinesin-related protein 3A (Fragment).- Rattus norvegicus - (Rat). |
| AAR20449 | AY426740 NID: - Rattus norvegicus |
| AAA57129 | RATAPMK NID: - Rattus norvegicus |
| Q9R189 | Munc13-4 protein. - Rattus norvegicus - (Rat). |
| A53621 | hydroxymethylglutaryl-CoA reductase (NADPH2) kinase (EC 2.7.1.109) - rat |
| AAQ91026 | AY387056 NID: - Rattus norvegicus |
| AAB95646 | RNU23443 NID: - Rattus norvegicus |
| TVRTK6 | ribosomal protein S6 kinase (EC 2.7.1.-), 70K - rat |

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| CATC RAT | Dipeptidyl-peptidase I precursor |
| S36126 | neural cell adhesion molecule L1 - rat |
| CAA73300 | RNLTBP2 NID: - Rattus norvegicus |
| Q9JM04 | Late gestation lung 2 protein.- Rattus norvegicus - (Rat). |
| Q7TPH4 | Dystrophin Dp7la.- Rattus norvegicus - (Rat). |
| CSRT | catalase (EC 1.11.1.6) - rat |
| Q62884 | Density-enhanced phosphatase-1 precursor |
| JC2130 | protein kinase (EC 2.7.1.37) - rat |
| BAA95006 | AB023896 NID: - Rattus norvegicus |
| Q8R4B6 | Testosterone-regulated prominin-related protein.- Rattus norvegicus - (Rat). |
| Q91XT9 | Ceramidase.- Rattus norvegicus - (Rat). |
| AAS75313 | AY5690l0 NID: - Rattus norvegicus |
| TMRTF1 | tropomyosin 1,-embryonic fibroblast - rat |
| O4RTD5 | cytochrome P450 2D5 - rat |
| Q8CFG6 | Calcium channel alpha-2 delta-2 subunit.- Rattus norvegicus |
| Q924N5 | Gonadotropin-regulated long chain acyl-CoA synthetase. |
| Q7TNY7 | Myosin VIIA and Rab interacting protein.- Rattus norvegicus |
| BAD10946 | AB038233 NID: - Rattus rattus |
| Q80WN5 | RSB-11-77.- Rattus norvegicus - (Rat). |
| S07085 | cystatin C precursor - rat (fragment) |
| Q7TP35 | Ab2-088 - Rattus norvegicus - (Rat). |
| O35814 | P60 protein.- Rattus norvegicus - (Rat). |
| CAD29097 | Chimeric DNA-directed DNA polymerase bf2-7a (EC 2.7.7.7) - Rat |

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| T46645 | sulfonylurea receptor 2B [imported] - rat |
| BAA75629 | AB002563 NID: - Rattus norvegicus |
| PCK9 RAT | Proprotein convertase subtilisin/kexin type 9 precursor (EC 3.4.21.-) Rattus norvegicus - (Rat). |
| Q91XW7 | Caspase 11.- Rattus norvegicus - (Rat). |
| A30411 | synapsin Ia - rat |
| Q9QWL9 | Dynamin IIIbb isoform. - Rattus norvegicus - (Rat). |
| CAD97562 | Sequence 1 from Patent EP1308514.- Rattus sp. |
| AAA62508 | RNU20796 NID: - Rattus norvegicus |
| T17187 | CL3AB protein - rat |
| S57953 | C4BP protein alpha chain precursor - rat |
| O70360 | Insulin receptor substrate 2 (fragment).- Rattus norvegicus (Rat) |
| JC7763 | neuronal leucine-rich repeat protein-3 - rat |
| Q9JIH3 | Delta kalirin-7.- Rattus norvegicus - (Rat). |
| BAA08621 | RATPK NID: - Rattus norvegicus |
| AAH61753 | BC061753 NID: - Rattus norvegicus |
| A43344 | synaptic vesicle protein SV2 - rat |
| AAC52355 | RNU40819 NID: - Rattus norvegicus |
| C40204 | Na+/H+ exchanging protein 4 - rat |
| AAB59692 | RATIRRB NID: - Rattus norvegicus |
| S39345 | unc-18 protein homolog, 67K - rat |
| CAA45743 | RNCYP3A1R NID: - Rattus norvegicus |
| JX0205 | long-chain-fatty-acid-CoA ligase (EC 6.2.1.3), brain [validated] - rat |
| AAL59007 | AY070268 NID: - Rattus norvegicus |

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|---|---|
| A31373 | calpactin I light chain - rat |
| BAC22515 | AB091379 NID: - Rattus norvegicus |
| JN0336 | N-methyl-D-aspartate receptor 1 precursor, splice form B - rat |
| S59964 | procollagen-lysine5-dioxygenase (EC 1.14.11.4) precursor- rat |
| AAD50905 | PAIRED IG-LIKE RECEPTOR-A1- Rattus norvegicus (Rat) |
| JC6508 | brahma related gene associated factor BAF60 b  protein - rat |
| O54772 | BAF60b.- Rattus norvegicus - (Rat). |
| A22952 | alpha-1 proteinase inhibitor III precursor - rat |
| BAA19517 | D84434 NID: - Rattus norvegicus |
| BAA05107 | RATCLCK2 NID: - Rattus rattus |
| Q80Z30 | Calmodulin-dependent  protein  kinase  phosphatase  N.-  Rattus norvegicus - (Rat). |
| AAB59698 | RATGLTPG NID: - Rattus norvegicus |
| CAA60630 | RNRNANE NID: - Rattus norvegicus |
| ERB2 RAT | Receptor protein-tyrosine kinase erbB-2 precursor |
| CAC60123 | Sequence 28 from Patent WO0153347. - Rattus rattus (Black rat) |
| Q8VDA0 | Ankyrin G107.- Rattus norvegicus - (Rat). |
| AAA41166 | RATFN3M2 NID: - Rattus norvegicus |
| AAQ96227 | AY383669 NID: - Rattus norvegicus |
| Q9ES75 | Proline-rich acidic protein.- Rattus norvegicus - (Rat). |
| AAH60515 | BC060515 NID: - Rattus norvegicus |
| BAD14292 | AB175782 NID: - Rattus norvegicus |
| AAA41653 | RATMYHCA NlD: - Rattus norvegicus |
| AAB31934 | S73559 NID: - Rattus sp. |

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| A46206 | voltage-gated sodium channel alpha subunit - rat (fragment) |
| Q7TN78 | Olfactory specific medium-chain acyl CoA synthetase. Rattus norvegicus - Rat |
| AAC63367 | AF093139 NID: - Rattus norvegicus |
| Q62977 | KRAB/zinc finger suppressor protein 1.- Rattus norvegicus - (Rat). |
| AAC52536 | RNU34841 NID: - Rattus norvegicus |
| S62785 | cytochrome P450 2C11 - rat |
| Q8CG40 | Putative alpha3-fucosyltransferase.- Rattus norvegicus - (Rat). |
| AAA41049 | RATCYPC NID: - Rattus norvegicus |
| Q9EQN5 | Antifreeze-enhancer binding protein AEP.- Rattus norvegicus - (Rat). |
| AAA41701 | RATNHEPSUL NID: - Rattus norvegicus |
| A40670 | nuclear envelope protein POM 121 - rat |
| O89049 | Thioredoxin reductase.- Rattus norvegicus - (Rat). |
| Q9Z1S6 | Melanocyte-specific protein 1.- Rattus norvegicus - (Rat). |
| S07390 | glucuronosyltransferase (EC 2.4.1.17) 3 precursor - rat |
| S16788 | probable reverse transcriptase - rat |
| AAG09802 | AF264765 NID: - Rattus norvegicus |
| Q80ZB6 | Purkinje cell espin isoform 2+.- Rattus norvegicus - (Rat). |
| Q9Z182 | Stretch-inhibitable nonselective channel (SIC).- Rattus norvegicus - (Rat). |
| AAQ96229 | AY383671 NID: - Rattus norvegicus |
| AAB632209 | AF008201 NID: - Rattus norvegicus |
| O54821 | Chloride channel 2. - Rattus norvegicus - (Rat). |

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| AAB05672 | RNU41853 NID: - Rattus norvegicus |
| T03096 | CDO protein - rat |
| A26294 | uncoupling protein - rat |
| AAC98728 | AF023303 NID: - Rattus norvegicus |
| Q8K1Q4 | ProSAPiP1 protein.- Rattus norvegicus - (Rat). |
| O88867 | Kynurenine 3-hydroxylase.- Rattus norvegicus - (Rat). |
| A46702 | methionyl aminopeptidase (EC 3.4.11.18) 2 - rat |
| S13875 | nicotinic acetylcholine receptor delta chain precursor - rat |
| Q8K3V0 | Osteoregulin-like protein.- Rattus norvegicus - (Rat). |
| Q8HW97 | 9 days embryo whole body cDNA, RIKEN full-length enriched library |
| A36330 | interferon regulatory factor 1 - rat |
| AAA18885 | RN10188 NID: - Rattus norvegicus |
| AAC52817 | RNU53420 NID: - Rattus norvegicus |
| A36304 | cytochrome P450 4A8 - rat |
| Q7TQ90 | Acl002.- Rattus norvegicus - (Rat). |
| GLK2 RAT | RAT Glutamate receptor. ionotropic kainate 2 precursor (Glutamate receptor 6) |
| S13913 | hexokinase (EC 2.7.1.1) III [similarity] - rat |
| O35804 | Janus protein tyrosine kinase 2 (Fragment).- Rattus norvegicus - (Rat). |
| CAB25334 | RAT PYRUVATE KINASE M INTRONLESS PROCESSED PSEUDOGENE |
| AAQ63060 | AY294938 NID:- Rattus norvegicus |

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| DCRTO | ornithine decarboxylase (EC 4.1.1.17) - rat |
| Q8K1Q5 | ProSAPiP2 protein.- Rattus norvegicus - (Rat). |
| BAA10926 | RATPI3KB NID: - Rattus norvegicus |
| ADDG RAT | Gamma adducin (Adducin-like protein 70) |
| Q7TPH3 | Dystrophin Dp71ab.- Rattus norvegicus - (Rat). |
| AMPN RAT | Aminopeptidase N (EC 3.4.11.2) (rAPN) (Alanyl aminopeptidase) |
| CAA55887 | RNTEST6 NID: - Rattus norvegicus |
| BAA04958 | RATRP NID: - Rattus norvegicus |
| AAF62175 | AF147452 NID: - Rattus norvegicus |
| O35825 | Inositol polyphosphate 4-phosphatase type II-beta.- Rattus norvegicus - (Rat). |
| Q920L2 | Flavoprotein subunit of succinate-ubiquinone reductase.- Rattus norvegicus |
| AAD24413 | AF057308 NID: - Rattus norvegicus |
| A44315 | cartilage oligomeric matrix protein precursor - rat |
| I58144 | corticotropin-releasing factor receptor - rat |
| A46216 | transcription factor Skn-1, splice form a - rat |
| A49413 | perilipin A - rat |
| AAH62000 | BC062000 NID: - Rattus norvegicus |
| O4RTLO | laurate omega-hydroxylase (EC 1.14.15.3) cytochrome P450 4A1 - rat |
| JN0710 | ubiquitin-like protein - mouse |
| AAC98727 | AF023302 NID: - Rattus norvegicus |
| I56606 | estrogen sulfotransferase isoform 3 - rat |

| | |
|---|---|
| KAPA RAT | cAMP-dependent protein kinase. alpha-catalytic subunit |
| O70142 | Sck (Fragment).- Rattus rattus (Black rat). |
| AAB48561 | RNU53367 NlD: - Rattus norvegicus |
| AAH63164 | BC063164 NID: - Rattus norvegicus |
| Q9JI56 | SNAP-29 protein.- Rattus norvegicus - (Rat). |
| Q91ZM1 | Transient receptor potential channel 4 alpha splice variant - Rattus norvegicus |
| CAD20352 | RNO427342 NID: - Rattus norvegicus |
| AAC19167 | AF043106 NID: - Rattus norvegicus |
| AAH59158 | BC059158 NID: - Rattus norvegicus |
| CAA69358 | RN2ARYLCO NID: - Rattus norvegicus |
| AAD31015 | AF130819 NID: - Rattus norvegicus |
| S38783 | integrin alpha chain - rat (fragment) |
| AAR95653 | AY435152 NID: - Rattus norvegicus |
| AAB06509 | RATJAPR NlD: - Rattus norvegicus |
| B32985 | somatotropin-binding protein precursor - rat |
| I57939 | taurine transporter - rat |
| 1DJGA2 | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase |
| AAH61717 | BC061717 NID: - Rattus norvegicus |
| Q924H6 | Testis-specific transporter TST1.- Rattus norvegicus - (Rat). |
| Q9QZP2 | Zinc finger protein.- Rattus norvegicus - (Rat). |
| BAA28746 | AB015042 NID: - Rattus norvegicus |
| S51005 | protein-tyrosine-phosphatase (EC 3.1.3.48) 2E - rat |
| Q9Z2A1 | Patched (Fragment).- Rattus norvegicus - (Rat). |

| | |
|---|---|
| JC4534 | cytochrome P450 4F6 protein - rat |
| AAH62402 | BC062402 NID: - Rartus norvegicus |
| Q9ET34 | Spliceosomal protein SAP155 (Fragment).- Rattus norvegicus |
| T17455 | translation initiation factor eIF-2 alpha chain kinase (EC 2.7.1.-) rat |
| S43506 | hypothetical protein - rat |
| Q9Z2I7 | SV2 related protein.- Rattus norvegicus (Rat ). |
| I73679 | estrogen sulfotransferase isoform 6 - rat |
| AAQ08184 | AF515735 NID: - Rattus norvegicus |
| S52097 | cytochrome P450III - rat |
| JH0695 | gamma-aminobutyric acid transporter protein 3 - rat |
| O35102 | Dihydroorotase (Fragment).- Rattus rattus (Black rat). |
| Q9JHE0 | Yotiao protein (Fragment).- Rattus norvegicus - (Rat). |
| JC5669 | Ca2+/calmodulin-dependent protein kinase kinase (EC 2.7.1.-) beta chain - rat |
| O54822 | Chloride channel 2.- Rattus norvegicus - (Rat). |
| AAH61840 | BC061840 NID: - Rattus norvegicus |
| I56563 | interleukin-3 receptor beta-subunit - rat |
| O54975 | Cytoplasmic aminopeptidase P.- Rattus norvegicus - (Rat). |
| ANRT | angiotensin precursor - rat |
| AAA85720 | RNU22952 NID: - Rattus norvegicus |
| Q8QZV1 | cGMP phosphodiesterase (Phosphodiesterase type 9).- Rattus norvegicus - (Rat). |
| Q9JK99 | Telomerase catalytic subunit (Fragment).- Rattus norvegicus - (Rat). |
| AAS66238 | AY539898 NID: - Rattus norvegicus |

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|---|---|
| A49681 | long-chain-fatty-acid beta-oxidation multienzyme complex alpha chain precursor |
| Q80Z29 | Pre-B-cell colony-enhancing factor.- Rattus norvegicus - (Rat). |
| Q9WV57 | Mpg-1 protein.- Rattus norvegicus - (Rat). |
| Q9Z2Y9 | Klotho.- Rattus norvegicus - (Rat). |
| S34226 | cyclin B - rat |
| I65309 | autoantigen p69 - rat |
| AAB48545 | RNU39943 NID: - Rattus norvegieus |
| Q7TP16 | Ccl-3.- Rattus norvegicus - (Rat). |
| Q9QZM5 | Eps8 binding protein.- Rattus norvegicus - (Rat). |
| Q8VIA9 | Ecotropic retrovirus receptor - Rattus norvegicus - (Rat). |
| GCRT | glucagon precursor - rat |
| A53433 | ribose-phosphate diphosphokinase (EC 2.7.6.1) 39K regulatory chain - rat |
| ET1R RAT | Endothelin-1 receptor precursor (ET-A).- Rattus norvegicus - (Rat). |
| A36275 | long-chain-fatty-acid-CoA ligase (EC 6.2.1.3), brain - rat |
| AAH06581 | BC006581 NID: - Mus musculus |
| AAR83745 | AY390379 NID: - Rattus norvegicus |
| Q923Z2 | Tropomyosin alpha isoform.- Rattus norvegicus - (Rat). |
| AAQ22768 | AY338396 NID: - Rattus rattus diardii |
| Q63636 | Natural killer cell protease 4.- Rattus norvegicus - (Rat). |
| AAH61812 | BC061812 NID: - Rattus norvegicus |
| Q64584 | Rat cytochrome P-450b (phenobarbital-inducible) - Rattus norvegicus - (Rat). |

| | |
|---|---|
| CAB35519 | Cytochrome P450.- Rattus norvegicus - (Rat). |
| AAC05303 | AF029105 NID: - Rattus norvegicus |
| ACM4 RAT | Muscarinic acetylcholine receptor M4.- Rattus norvegicus - (Rat). |
| Q9EPT7 | Prothrombinase FGL2.- Rattus norvegicus - (Rat). |
| Q80WF4 | GRP78 binding protein.- Rattus norvegicus - (Rat). |
| AAA41654 | RATMYHCB NID: - Rattus norvegicus |
| Q8K4V4 | PDZ protein Mrt1A.- Rattus norvegicus - (Rat). |
| B56021 | probable cell division control protein p55CDC - rat |
| I56531 | beta-adrenergic-receptor kinase (EC 2.7.1.126) 1 - rat |
| Q7TP91 | Abl-205 - Rattus norvegicus - (Rat). |
| JH0819 | calreticulin precursor - rat |
| AAK71880 | AY039651 NID: - Rattus norvegicus |
| A42811 | nuclear RNA helicase (DEAD family) homolog - rat |
| A28055 | K-kininogen, LMW 1 precursor - rat |
| Q924K5 | Myocilin - Rattus norvegicus - (Rat). |
| O08963 | Protocadherin 4.- Rattus rattus (Black rat). |
| A26685 | cytochrome P450 2C11 - rat |
| CAC34480 | RNO306427 NID: - Rattus norvegicus |
| AAF15588 | AF188333 NID: - Rattus norvegicus |
| JC1285 | protein-tyrosine-phosphatase (EC 3.1.3.48), receptor type alpha precursor - rat |
| T10772 | calpastatin - rat |
| AAD33896 | AF143545 NID: - Rattus norvegicus |
| Q7TPJ8 | Ac2-143.- Rattus norvegicus - (Rat). |

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|---|---|
| AAH61839 | BC06l839 NID: - Rattus norvegicus |
| Q80WA5 | Sodium myo-inositol transporter 1.- Rattus norvegicus - (Rat). |
| AAL26912 | AF320054 NID: - Rattus norvegicus |
| S12736 | hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5), cytosolic - rat |
| Q9EPH2 | Mac-MARCKS protein.- Rattus norvegicus - (Rat). |
| JE0344 | contraception associated protein 1 - rat |
| AAC83348 | AF085693 NID: - Rattus norvegicus |
| CAC16598 | RNO277748 NID: - Rattus norvegicus |
| GBP2 RAT | Interferon-induced guanylate-binding protein 2 (GTP-binding protein 2) |
| Q9Z1J7 | Sodium-dependent neutral amino acid transporter, ASCT2. - Rattus norvegicus |
| B39667 | brain-derived neurotrophic factor receptor precursor, splice form T1 - rat |
| A36205 | mitochondrial processing peptidase (EC 3.4.24.64) alpha chain precursor |
| Q7TQ77 | Ac1288.- Rattus norvegicus - (Rat). |
| T17367 | potassium channel protein elk1 - rat |
| AAA41880 | RATPKL NID: - Rattus norvegicus |
| Q9Z0R8 | Putative taste receptor TR1 (Fragment).- Rattus norvegicus - (Rat). |
| Q810H6 | Disrupted-in-schizophrenia-1.- Rattus norvegicus - (Rat). |
| S11737 | resistance protein Mx3, interferon-regulated - rat |
| Q8K451 | GABAB-related G-protein coupled reccptor.- Rattus norvegicus |
| CAC14912 | RNO296090 NID: - Rattus norvegicus |

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|---|---|
| MK06 RAT | Mitogen-activated protein kinase 6 |
| O70498 | Nuclear RNA helicase.- Rattus norvegicus - (Rat). |
| A36116 | prolactin receptor 2 precursor - rat |
| A27274 | ribophorin I precursor - rat |
| AXN1 RAT | Axin 1 protein (Axis inhibition protein 1) (rAxin).- Rattus norvegicus - (Rat). |
| Q8K1Q1 | Vesicular glutamate transporter 3.- Rattus norvegicus - (Rat). |
| AAH61996 | BC061996 NID: - Rattus norvegicus |
| JC5261 | salt-tolerant protein - rat |
| AAS66230 | AY539890 NID: - Rattus norvegicus |
| S49162 | ZG-21p protein - rat |
| FXM1 RAT | Forkhead box protein M1 (Winged helix factor from INS-1 cells) |
| AAN33055 | AF538953 NID: - Rattus norvegicus |
| JC4841 | regeneration associated serpin-1 precursor - rat |
| MK10 RAT | Mitogen-activated protein kinase 10 |
| Q8SEZ7 | NADH dehydrogenase subunit 2 (Fragment).- Rattus norvegicus |
| AAC63994 | AF058795 NID: - Rattus norvegicus |
| AAC52771 | RNU4001 NID: - Rattus norvegicus |
| NUCL RAT | Nucleolin (Protein C23).- Rattus norvegieus - (Rat). |
| Q8CHN8 | Mannose-binding protein associated serine protease-1- Rattus norvegicus |
| Q9JJ09 | Type IIb sodium-phosphate transporter.- Rattus norvegicus |
| O08623 | PKC-zeta-interacting protein (ZIP).- Rattus norvegicus - (Rat). |
| Q99N97 | H-caldesmon (Fragment).- Rattus norvegicus - (Rat). |

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|---|---|
| Q91ZV2 | Endothelial and smooth muscle cell-derived neuropilin-like protein - Rat |
| Q63614 | Tyrosine kinase receptor (Fragment).- Rattus norvegicus - (Rat). |
| Q63778 | Hypothetical protein.- Rattus norvegicus - (Rat). |
| AAA75166 | RNU21683 NID: - Rattus norvegicus - (Rat). |
| T17156 | CL1BB protein - rat |
| JC5614 | RNB6 protein - rat |
| I59331 | thyrotropin-releasing hormone degrading enzyme (EC 3.4.11.-) rat |
| Q91XR7 | Amino acid transporter system N2.- Rattus norvegicus - (Rat). |
| A55622 | cytochrome P450 C11B3 - rat |
| DYMSD2 | dopamine receptor D2 - mouse |
| Q91XJ1 | Bcl-2 -interacting coiled-coil protein beclin.- Rattus norvegicus |
| CAA72533 | RNECTOATP NID: - Rattus norvegicus |
| SRE1 RAT | Sterol regulatory element binding protein-l (SREBP-1) |
| GB12 RAT | Guanine nucleotide-binding protein, alpha-12 subunit (G alpha 12) - Rat |
| I51883 | alpha-2B-adrenergic receptor - rat |
| JC2054 | complement regulatory protein. 5I2 antigen precursor - rat |
| S36327 | clathrin assembly protein APl80 long form - rat |
| A47503 | epoxide hydrolase (EC 3.3.2.3), cytosolic - rat |
| CAD55407 | RNO512838 NID: - Rattus norvegicus |
| Q9JHZ9 | Amino acid system N transporter.- Rattus norvegicus - (Rat). |
| S51605 | receptor-like tyrosine kinase Ehk-2 - rat |
| A46595 | tricarboxylate transport protein precursor. mitochondrial - rat |

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|---|---|
| O35260 | NAC-1 protein.- Rattus norvegicus - (Rat). |
| S68200 | glucuronosyltransferase (EC 2.4.1.17) precursor - rat |
| I58099 | gene PZX3 protein - rat |
| JH0672 | brain factor 1 protein - rat |
| AAB38506 | RNU80054 NID: - Rattus norvegicus |
| A39030 | androgen-binding protein 1 precursor - rat |
| Q7TP48 | Ab2-305 - Rattus norvegicus - (Rat). |
| SAHH RAT | Adenosylhomocysteinase (EC 3.3.1.1) |
| AAQ91067 | AY387097 NID: - Rattus norvegicus |
| Q7TMD5 | Nuclear protein UKp83.- Rattus norvegicus - (Rat). |
| B38656 | vacuolar proton pump 116K chain - rat |
| Q7TMA9 | Acl262 (Aal249) (Ac1-114) (Ac2-069) (Ab2-196) (Ab1-341) (Ba2-693) - Rat |
| Q9EQ23 | C-kit receptor (Fragment).- Rattus norvegicus - (Rat). |
| XURT | acetyl-CoA C-acyltransferase (EC 2.3.1.16), mitochondrial - rat |
| Q9ESN0 | Niban.- Rattus norvegicus - (Rat). |
| I59282 | diacylglycerol kinase (EC 2.7.1.107) gamma - rat |
| JC1450 | Fibroblast growth factor receptor 4 - rat |
| O70177 | Carboxylesterase precursor (EC 3.1.1.1).- Rattus norvegicus |
| AAA40950 | RATCP450 NID: - Rattus norvegicus |
| A53047 | 6-phosphofructokinase, (EC 2.7.1.11) - rat |
| A30314 | protein kinase C (EC 2.7.1.-) zeta - rat |
| RIP2 RAT | RIM binding protein 2 (RIM-BP2).- Rattus norvegicus - (Rat). |
| B47417 | insulin receptor-related receptor, secreted splice form 2 precursor - |

| | rat |
|---|---|
| Q99PW1 | Protein tyrosine kinase c-Yes (Fragment).- Rattus norvegicus |
| A54139 | Na+/Ca2+ exchanging protein NCX2 - rat |
| Q8VHY4 | Transcription factor GATA-2.- Rattus norvegicus - (Rat). |
| B39816 | tropomyosin 3. fibroblast - rat |
| CTRTP | corticotrophin / lipotropin precursor - rat |
| Q9WUV7 | Serine/threonine specific protein phosphatase (EC 3.1.3.16) |
| Q8VHU3 | Hypothetical RNA binding protein RDA288.- Rattus norvegicus |
| A34695 | axonal glycoprotein TAG-1 precursor- rat |
| XXRTAC | acetyl-CoA C-acetyltransferase (EC 2.3.1.9) precursor, mitochondrial - rat |
| Q80YG1 | Phosphoserine aminotransferase.- Rattus norvegicus - (Rat). |
| Q9ER33 | Cyclic nucleotide-gated channel 2b.- Rattus norvegicus - (Rat). |
| CHRTD1 | potassium channel protein drk1 - rat |
| O54771 | BAF60b (Fragment).- Rattus norvegicus - (Rat). |
| AAC12270 | AF026554 NID: - Rattus norvegicus |
| Q80W59 | Histidine-rich calcium binding protein.- Rattus norvegicus - (Rat). |
| A39257 | cytochrome P450 2C22 - rat |
| Q9Z311 | Nuclear receptor binding factor-1.- Rattus norvegicus - (Rat). |
| S41627 | probable anti-mullerian hormone receptor - rat |
| RGHYA2 | GTP-binding regulatory protein Gs alpha-2 chain |
| T42260 | guanylate cyclase (EC 4.6.1.2) G precursor- rat |
| A34625 | gamma-aminobutyric acid/benzodiazepine receptor type A delta chain precursor |

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|---|---|
| O88292 | Protein C inhibitor.- Rattus norvegicus - (Rat). |
| JC4163 | DNA-binding protein 5E5 - rat |
| Q9WVG4 | Gliosarcoma-related antigen MIDAl (Fragment).- Rat. |
| QYRTGP | phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) cytosolic - Rat |
| Q62916 | Metabotropic glutamate receptor 4b.- Rattus norvegicus - (Rat). |
| AAQ93383 | AY393999 NID: - Rattus norvegicus |
| Q8K3F0 | Na+ dependent amino acid transporter ASCT2.- Rattus norvegicus - (Rat). |
| SMS1 RAT | Phosphatidylcholine:ceramide cholinephosphotransferase 1 |
| Q99PJ8 | Zinc finger protein HIT-10.- Rattus norvegicus - (Rat). |
| JU0133 | translation elongation factor eEF-1 alpha chain - Chinese hamster |
| S34780 | gelatinase A (EC 3.4.24.24) precursor - rat |
| Q9EQH5 | Ribeye.- Rattus norvegicus - (Rat). |
| MUCL RAT | Intestinal mucin-like protein (MLP) (Fragment).- Rattus norvegicus |
| A27671 | spectrin alpha chain. nonerythroid - rat (fragment) |
| Q99NC1 | Nuclear protein UKp68.- Rattus norvegicus - (Rat). |
| Q9QYU1 | PxF protein.- Rattus rattus (Black rat). |
| Q810W9 | CASK-interacting protein CIP98.- Rattus norvegicus - (Rat). |
| A41028 | aldehyde dehydrogenase [NAD] (EC 1.2.1.3) 4 microsomal [similarity] - rat |
| Q99MZ8 | LASP-1 Rattus norvegicus - (Rat). |
| A56517 | nucleoporin Nup98 - rat |
| A41158 | dipeptidyl-peptidase I (EC 3.4.14.1) precursor - rat |

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|---|---|
| I60373 | uKATP-1 rat |
| JC5803 | ring finger protein, BFP - rat |
| Q64718 | Elongation factor-1 alpha.- Rattus norvegicus - (Rat). |
| A35730 | H+ transporting two-sector ATPase (EC 3.6.3.14) alpha 4 chain precursor - rat |
| I66868 | cdc25B - rat |
| JC5701 | ErbB kinase activator alpha1, brain and thymus - rat |
| A48189 | sodium/phosphate cotransport protein. renal cortex - rat |
| Q63158 | Zinc finger protein - Rattus norvegicus - (Rat). |
| Q9ES02 | Osteoregulin.- Rattus norvegicus - (Rat). |
| A25047 | beta-glucuronidase (EC 3.2.1.31) precursor - rat |
| TMRBB | tropomyosin beta chain, skeletal muscle [validated] - rabbit |
| T42218 | slit-1 protein homolog - rat |
| I55462 | retinol dehydrogenase (EC 1.1.1.105) type II, 29K - rat |
| Q4RTPB | cytochrome P450 2B1 - rat |
| A53582 | Na+/glucose cotransporter SGLT1 - rat |
| I63224 | clathrin-associated adaptor protein - rat |
| Q8CG04 | cAMP-specific phosphodiesterase PDE4D7.- Rattus norvegicus |
| Q9QZX1 | Nucleolin-related protein NRP.-Rattus norvegicus - (Rat). |
| TDRTLT | leukocyte common antigen precursor, splice form 4 - rat |
| Q9EPG9 | ABC transporter, white homologue.- Rattus norvegicus - (Rat). |
| A29514 | muscarinic acetylcholine receptor M1 - rat |
| Q9R1K3 | P2X3b receptor (P2X purinoceptor) (ATP receptor) (Purinergic receptor) Rat |

| | |
|---|---|
| S23431 | choline transport protein - rat |
| CAD35208 | Sequence 52 from Patent WO0226984.- Rattus sp. |
| Q9Z1R8 | Guanine nucleotide-binding protein.- Rattus norvegicus - (Rat). |
| A42499 | mullerian inhibiting factor precursor - rat |

TABLE 4.2. **Proteins in abnormal CSF not present in normal CSF.**

| PROTEIN ID | CHEMICAL NAME |
|---|---|
| S16233 | nitric oxide synthase - rat |
| AAQ96265 | AY383707 NID: - Rattus norvegicus |
| AAR95664 | AY480031 NID: - Rattus norvegicus |
| S37146 | myosin 1 heavy chain - rat |
| UBRTA | tubulin alpha chain - rat |
| AAS66264 | AY539924 NID: - Rattus norvegicus |
| S57329 | tuberous sclerosis 2 homolog - rat |
| 3FYGA | mu class tetradeca - (3-fluorotyrosyl)-glutathione s-transferase of isoenzyme |
| Q9QXL9 | Dynamin IIIbb isoform - (Rat). |
| Q91ZD8 | Synaptojanin 2B2. - Rattus norvegicus - (Rat). |
| CAF05438 | AX95162 NID: - Rattus norvegicus |
| A53933 | myosin I myr 4 - rat |
| Q7TMC7 | Ab2-417 (Cc1-8).- Rattus norvegicus - (Rat). |
| C41539 | kinesin light chain C - rat |
| T17158 | CL2AB protein - rat |
| O35303 | Dynamin-like protein (DLP1 splice variant 3). - (Rat). |
| Q7TNX0 | Liver regeneration-related protein LRRG03. - (Rat). |
| DNM1 RAT | DNA (cytosine-5)-methyltransferase 1 |
| A32202 | prostaglandin-D synthase - rat |
| T03301 | rab3 effector protein Rim - rat |

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|---|---|
| Q9ERD4 | Ankyrin repeat-rich membrane-spanning protein. - (Rat). |
| A45439 | myosin I heavy chain - rat |
| I58166 | rabphilin-3A - rat |
| S52517 | myosin I heavy chain - rat |
| S49163 | transferrin precursor - rat |
| Q8VHT3 | L-type calcium channel alpha-lc subunit. - (Rat). |
| Q7TN17 | Kinesin-like protein KIF15. -(Rat). |
| O35774 | 75 kDa fibrous sheath protein. - (Rat). |
| Q9QWG5 | Inositol polyphosphate 4-phosphatase type II-alpha. |
| Q8VD81 | cAMP-specific phosphodiesterase isoform PDE4B4. - rat |
| A40439 | UBF transcription factor, long form - rat |
| T14050 | protein kinase beta, myotonic dystrophy-associated - rat |
| S02180 | secretogranin II - rat |
| Q9Z200 | APS protein.- Rattus norvegicus - (Rat). |
| S68453 | sodium channel protein SNS - rat |
| Q62744 | Alpha actinin.- Rattus norvegicus - (Rat). |
| Q8R4C9 | RNA-binding protein staufen splice form B. - (Rat). |
| Q9QUI9 | CENTAURIN beta.- Rattus norvegicus - (Rat) |
| S70581 | dihydropyrimidinase  rat |
| A53300 | ribosomal protein S6 kinase (EC 2.7.-.-) II - rat |
| ACC8 RAT | Sulfonylurea receptor 1. - (Rat). |
| Q812D9 | DRE1.- Rattus norvegicus - (Rat) |
| Q9JIG0 | Kalirin-7c isoform.- (Rat). |
| T42759 | Munc13-3 protein - rat |

| | |
|---|---|
| 5FWGA | tetra-(5-fluorotryptophanyl) -glutathione transferase mu class (EC 2.5.1.18) mutant 4 tryptophans M |
| Q9EPH6 | Uncoupling protein UCP-4, isoform b .- (Rat). |
| A41009 | glutuminase (EC 3.5.1.2) precursor, mitochondrial - rat |
| S06017 | neuraxin - rat |
| Q9R019 | Checkpoint kinase Chk2. - (Rat). |
| Q9JJ32 | NBC-like protein. - (Rat). |
| CAA04248 | RNAJ696 NID: - Rattus norvegicus |
| AAC06273 | RNU69278 NID: - Rattus norvegicus |
| AAA40724 | RATALS NID: - Rattus norvegicus |
| B54091 | rab GDP dissociation inhibitor beta - rat |
| Q9JJ40 | PDZ domain containing protein. - (Rat). |
| Q63560 | Stop protein. Rattus norvegicus - (Rat). |
| Q9EQV7 | Nadrin. Rattus norvegicus - (Rat). |
| Q63939 | Myosin heavy chain (Fragment).- Rattus sp. |
| AAC72251 | RNU48247 NID: - Rattus norvegicus |
| A31982 | Ca2+ transporting ATPase (EC 3.6.3.8) RB2-5, brain |
| RL10 MOUSE | 60S ribosomal protein L10 (QM protein homolog) - (Mouse), and (Rat) |
| ANX2 RAT | Annexin A2 (Annexin II) (Lipocortin II) |
| A28807 | protein disulfide-isomerase ER60 precursor - rat |
| I56581 | dnaK-type molecular chaperone grp75 precursor - rat |
| Q9ES53 | UFD1.- Rattus norvegicus - (Rat). |
| Q7TSC6 | Jacob - Rattus norvegicus - (Rat). |

| | |
|---|---|
| A53742 | calponin, acidic - rat |
| Q9QVN4 | R NR-CaM 22 protein. Rattus sp. |
| KIRTPL | pyruvate kinase, hepatic splice form L - rat |
| KLC1 RAT | Kinesin light chain 1 (KLC 1) - (Rat). |
| LURT3 | annexin III - rat |
| A48157 | renal transcription factor Kid-1 - rat |
| Q810Y4 | Alpha 1 type XXIII collagen. - (Rat) |
| A54766 | metastasis-associated protein mta-1 - rat |
| S15074 | calpastatin - rat |
| Q9EQR2 | Alkyl-dihydroxyacetonephosphate synthase - (Rat). |
| T14327 | alpha-latrotoxin receptor 3, calcium-independent |
| S34656 | amine oxidase, precursor - rat |
| Q7TMB1 | AC1147 (Aa1011) (Aa1262) (Ab2-057).- (Rat). |
| S27267 | lamin A - rat |
| JN0065 | ribophorin II precursor - rat |
| Q920L0 | SLP-76 adaptor protein.- Rattus norvegicus - (Rat). |
| B29231 | glutathitone transferase (EC 2.5.1.18) Yb4 - tat |
| O55153 | Calpastatin,- Rattus norvegicus - (Rat). |
| PC7034 | Na+ bicarbonate contranspotter - rat |
| Q9EPI6 | Jacob protein.- Rattus norvegicus - (Rat). |
| Q8R471 | Kinesin-related protein 3B (Fragment) - (Rat). |
| CAD34650 | Sequence 5 from Patent WO0224187. - (Rat) . |
| Q7TNJ9 | stonin.- Rattus norvegicus - (Rat). |
| A56039 | GTPase-activating protein Gap1 (m) - rat |

| | |
|---|---|
| S11508 | D100 protein - rat |
| Q8K4G7 | Rho GTPase activating protein 4. - (Rat). |
| A36001 | choline-phosphate cytidylyltransferase - rat |
| JX0334 | cytochrome P450 SA RL33 - rat |
| Q9JHX9 | Fibroblast growth factor receptor 3 - (Rat). |
| Q7TQ08 | Scinderin.- Rattus norvegicus (Rat). |
| Q925V4 | Cyclooxygenase-2.- Rattus norvegicus - (Rat). |
| I67945 | 3',5'-cyclic-nucleotide phosphodiesterase - rat |
| JC7186 | alpha-actinin-4 - rat |
| Q8K4M9 | Oxysterol-binding protein.- Rattus norvegicus - (Rat). |
| Q8CFM6 | Hyaluronan receptor for endocytosis HARE precursor |
| S29840 | fibroblast growth factor receptor 1 - rat |
| O89000 | Dihydropyrimidine dehydrogenase (EC 1.3.1.2). - (Rat) |
| AAC24984 | AF027571 NID: - Rattus norvegicus |
| Q923H3 | Insulin receptor tyrosine kinase substrate protein p53 - rat |
| T09222 | exocyst complex protein Exo84 - rat |
| A55913 | transcytosis-associated protein p115 - rat |
| A60560 | formyltetrahydrofolate dehydrogenase (EC 1.5.1.6) / aldehyde dehydrogenase (NADP) (EC 1.2.1.4) - Rat |
| T17365 | serine/threonine protein kinase TAO1. - rat |
| Q8CFG5 | Calcium channel alpha-2 delta-3 subunit - (Rat) . |
| A499128 | cell-fate determining gene Notch2 protein - rat |
| Q7TP30 | Ac2-155.- Rattus norvegicus - (Rat) . |
| A46691 | E-box-binding protein beta form - rat |

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|---|---|
| AAB37540 | RNU384181 NID: - Rattus norvegicus |
| I53417 | type I serine-threonine kinase receptor - rat |
| SCP1 RAT | Synaptonemal complex protein 1 (SCP-1 protein) - (Rat). |
| MPT1 RAT | Protein phosphatase 1 regulatory subunit 12A |
| A33569 | alcohol sulfotransferase (EC 2.8.2.2) - rat |
| Q8K4C8 | Frizzled-9.- Rattus norvegicus - (Rat). |
| EF2 RAT | Elongation factor 2 (EF-2) .- Rattus norvegicus - (Rat). |
| WZRTRS | argininosuccinate lyase (EC 4.3.2.1) - rat |
| KPY2 RAT | Pyruvate kinase, M2 isozyme (EC 2.7.1.40) - (Rat). |
| S54342 | protein-tyrosine-phosphatase, receptor type N precursor - rat |
| NFM RAT | Neurofilament triplet M protein (160 kDa neurofilament protein) - (neurofilament medium polypeptide) |
| A38135 | ADP-ribosylarginine hydrolase - rat |
| Q7TP95 | Ab1-351.- Rattus norvegicus - (Rat). |
| S57901 | estradiol 17beta-dehydrogenase (EC 1.1.1.62) - rat |
| Q9ES38 | Bile acid CoA ligase.- Rattus norvegicus - (Rat). |
| GERTX1 | matrix glycoprotein SC1 precursor - rat |
| RGHYO1 | GTP-binding regulatory protein Go alpha chain, splice form alpha-1 - golden hamster |
| AATC RAT | Aapartate aminotransferase, cytoplasmic |
| Q80YN5 | Histone acetyltransferase.- Rattus norvegicus (Rat). |
| TVRTRF | protein kinase raf-1 (EC 2.7.1.-) - rat |
| CAA35934 | RNA1B NID: - Rattus norvegicus |
| Q62775 | SH3 domain binding protein.- Rattus norvegicus - (Rat). |

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|---|---|
| Q8CJ98 | Sodium channel associated protein 1B. - (Rat) . |
| Q9ESY8 | Staufen isoform Stau-I6.- Rattus norvegicus - (Rat). |
| A30303 | interferon-related protein PC4 - rat |
| S42880 | amyloid pracursor-like protein - rat |
| Q9ERD5 | Synaptotagmin 13.- Rattus norvegicus - (Rat). |
| AAC52383 | RNU04998 NID: - Rattus norvegicus |
| A48508 | cyclic-nucleotide phosphodiesterase, cGMP-inhibited - rat |
| S09583 | peptidylglycine monooxyganase B precursor - rat |
| NAC1 RAT | sodium/calcium exchanger 1 precursor - (Rat). |
| ATB2 RAT | Plasma membrane calcium-transporting ATPase 2 |
| Q9WVJ6 | Tissue-type transglutaminase. - (Rat). |
| PCK5 RAT | Proprotein convertase subtilisin/kexin type 5 precursor |
| AAC32866 | AF083341 NID: - Rattus norvegicus |
| Q99JE4 | Guanine nucleotide release/exchange factor - (Rat) . |
| JC7265 | neprilysin (EC 3.4.24.11) II - rat |
| Q9QUL6 | N-ethylmaleimide sensitive factor.- (Rat) |
| B49555 | enhancer of split homolog R-esp2 - rat |
| A29952 | alpha-1 proteinase inhibitor III precursor - rat |
| AAD37861 | AF142759 NID: - Rattus norvegicus |
| SUHA RAT | Alcohol sulfotransferase A |
| T17200 | CL3BC protein - rat |
| I55476 | growth potentiating factor - rat |
| T17188 | CL3AC protein - rat |
| Q9QYJ6 | PDE10A2 (EC 3.1.4.17) .- Rattus norvegicus - (Rat) . |

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|---|---|
| Q9WU06 | Pervin.- Rattus norvegicus - (Rat). |
| A54065 | sodium transport protein gamma chain - rat |
| A55620 | apical endosomal protein precursor - rat |
| ATHA RAT | Potassium-transporting ATPase alpha chain 1 |
| Q63477 | Protein tyrosine phosphatase epsilon C - (Rat). |
| Q8K4S6 | Centrosomal protein CG-NAP (Fragment) - (Rat). |
| Q9R120 | Heat shock factor 2.- Rattus norvegicus - (Rat). |
| 1B5A | ferrocytochrome b5 as axial ligands - E.Coli |
| Q99PD9 | Down-regulated in adenoma DRA (Fragment) - (Rat) . |
| I60173 | adenine nucleotide translocator - rat |
| AAH60533 | BC060533 NID: - Rattus norvegicus |
| Q9JKZ4 | Thioredoxin reductase 1.- Rattus norvegicus - (Rat). |
| Q8K4H3 | TUC-4b.- Rattus norvegicus - (Rat). |
| A40452 | keratin 21, type I, cytoskeletal - rat |
| AAF26878 | T cell receptor V delta 6 (fragment).- (Rat). |
| Q925G1 | Hepatoma-derived growth factor 3. - (Rat) . |
| Q9WVT5 | Sterol 12alpha-hydroxylase P450. - (Rat). |
| Q8R5I3 | Ras guanine nucleotide releasing protein 4 variant 1- rat |
| Q9Z1I2 | P90 S6 kinase (Fragmant).- Rattus norvegicus - (Rat). |
| Q924M0 | GABA type B receptor 1f- Rattus norvegicus - (Rat) . |
| Q91YB4 | Putative RNA binding protein 1 (Fragment). - (Rat). |
| T31096 | cyclin G-associated kinase GAK - rat |
| Q35470 | cAMP-specific phosphodiesterase. - (Rat). |
| S21347 | hypothetical protein 3 - rat |

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|---|---|
| Q924Y2 | Na+/Ca2+ exchanger.- Rattus norvegicus - (Rat). |
| I84692 | nuclear orphan receptor HZF-3 - rat |
| Q80WX7 | Soluble guanylyl cyclase beta 2 subunit. - (Rat). |
| S38361 | calpain (EC 3.4.22.17) II large chain - rat |
| GDIA RAT | Rab GDP dissociation inhibitor alpha (Rab GDI alpha) - rat |
| Q8CGZ2 | Afadin-and alpha actinin-binding protein ADIP. - (Rat). |
| BAA03369 | RATCLS4A NID: - Rattus norvegicus |
| AAF08536 | AF1E0350 NID: - Rattus norvegicus |
| A33837 | insulin-like growth factor I receptor precursor - rat |
| Q920F3 | Nuclear RNA binding protein SLM-1. - (Rat). |
| S52913 | nuclear receptor Rev-ErbA beta - rat |
| S16385 | macrophage colony-stimulating factor 1 receptor precursor - rat |
| A27449 | T-cell surface glycoprotein CD4 precursor - rat |
| URRTAP | peptidylglycine monooxygenase precursor - rat |
| O35788 | Cyclic nucleotide-gated channel beta subunit. - (Rat) . |
| A32140 | Steroid 15beta-monooxygenase, cytochrome P450 2C12 - rat |
| Q9JK64 | Multidrug resistance protein 1a. - (Rat). |
| AAQ91035 | AY387065 NID: - Rattus norvegicus |
| P70591 | KRAB-zinc fingor protein KZF-2. - (Rat). |
| ANX1 RAT | Annexin A1 (Annexin I) (Lipocortin I) (Calpactin II) |
| Q9Z2M4 | Putative peroxisomal 2,4-dienoyl-CoA reductase. - (Rat) |
| S23399 | chloride channel protein 2 - rat |
| Q8K3G2 | Hypertrophied skeletal muscle protein GTF3 (Fragment) - rat |
| AAA41875 | RATPKCII NID: - Rattus norvegicus |

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|---|---|
| A36087 | histidine ammonia-lyase (EC 4.3.1.3) - rat |
| Q924Z9 | Netrin-1.- Rattus norvegicus - (Rat). |
| Q9JMB2 | Type II brain 4.1 minor isoform. - (Rat). |
| Q9ERS3 | Voltage-gated calcium channel alpha2/delta-1 subunit-rat |
| Q8R455 | Cold/menthol receptor 1.- Rattus norvegicus - (Rat). |
| Q925V9 | Cationic amino sold transporter-2A. - (Rat). |
| KHRTD | cathepsin D (EC 3.4.23.5) precursor - rat |
| Q9EQR7 | Cyclic AMP phosphodiesterase PDE4A10 (Fragment) - (Rat) |
| Q99N33 | R8f DNA-binding protein.- Rattus norvegicus - (Rat). |
| AAS21245 | AY462093 NID: - Rattus norvegicus |
| Q99NH5 | GATA-3.- Rattus norvegicus - (Rat). |
| CAC18955 | Sequence 1 from Patent WO0068386.- Rattus sp. |
| P70593 | A-kinase anchoring protein AKAP15O. - (Rat). |
| S53457 | dominant autoantigen gp 330 - rat (fragment) |
| I54195 | parathyroid hormone/parathyroid hormone related-peptide receptor - rat |
| T09054 | capsaicin receptor - rat |
| KAP0 RAT | cAMP-dependent protein kinase type 1-alpha regulatory chain - (Rat) |
| Q7TP36 | Ab2-404 - Rattus ncrvagicus - (Rat). |
| AAA75407 | RATPTPP1 NID: - Rattus norvegicus |
| S43677 | Protein tyrosine kinase JAK3 - rat |
| Q9R1J8 | Leprecan.- Rattus norvegicus - (Rat). |
| E49164 | chromogranin-B - rat (fragment) |

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|---|---|
| CLAT RAT | Choline O-acetyltransferase (CHOACTase) (Rat) . |
| Q7TPK0 | Ac2-125 - Rattus norvegicus - (Rat). |
| T10799 | sucrose alpha-glucosidase / oligo-1,6-glucosidase - rat |
| O88941 | Glycoprotein processing glucosidase I. - (Rat). |
| Q9QYD5 | Putative sodium bicarbonate cotransporter. - (Rat) . |
| A54756 | Isocitrate dehydrogenase (NADP) cytosolic - rat |
| UCP1 RAT | Mitochondrial brown fat uncoupling protein 1 - (Rat). |
| Q9JKC1 | Butyrylcholinesterase - Rattus norvegicus - (Rat). |
| S33777 | hepsin (EC 3.4.21.-) - rat |
| Q7TQ86 | Ac1158.- Rattus norvegicus - (Rat). |
| Q9Z327 | Synaptopodin.- Rattus norvegicus (Rat). |
| Q8R560 | Ankyrin-repeat protein.- Rattus norvegicus - (Rat). |
| 9QWJ1 | Retinoic acid receptor alpha 2 isoform. - (Rat). |
| S59927 | 14-3-3 protein, theta subtype - rat |
| JH0562 | metabotropic glutamate receptor 3 precursor - rat |
| Q9QZP9 | Septin-like protein SLP-b.- Rattus norvegicus - (Rat). |
| JC5399 | dual leucine zipper kinase (EC 2.7.-.-) - rat |
| S41053 | 1D-myo-inositol-trisphosphate 3-kinase B - rat |
| C24639 | Na+/K+ exchanging ATPase (EC 3.6.3.9) alpha-3 chain - rat |
| Q8VIH3 | Concentrative Na+-nucleoside cotransporter protein - rat |
| AAD08899 | AF041838 NID: - Rattus norvegicus |
| R5HU7A | ribosomal protein L7a, cytosolic - human |
| O08619 | Factor XIIIa.- Rattus norvegicus - (Rat). |
| A55915 | guanylate cyclase (EC 4.6.1.2) 2E precursor - rat |

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|---|---|
| SUHS RAT | Alcohol sulphotransferase - (Rat). |
| T14154 | serine/threonine protein kinase ANPK, nuclear - rat |
| S34667 | inward rectifier potassium channel - rat |
| UD12 RAT | UDP-glucuronosyltransferase 1-2 precursor, microsomal |
| PRS7 RAT | 26S protease regulatory subunit 7 (MSS1 protein) - (Rat) |
| A28821 | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase I - rat |
| A31870 | amine oxidase (flavin-containing) (EC 1.4.3.4) B - rat |
| Q9JMB3 | Type II brain 4.1.- Rattus norvegicus (rat). |
| JC5285 | carbonyl reductase (NADPH2) , noninducible - rat |
| S12061 | hexokinase (EC 2.7.1.1) type IV - rat |
| P97842 | 5-hydroxytryptamine7 receptor isoform c - (Rat) |
| AAA41648 | RATMYHAB2 NID: - Rattus norvegicus |
| S47647 | Nitric oxide synthase (EC 1.14.13.39) - rat |
| 1B9YC | phosducin, chain C - rat (fragments) |
| MACS RAT | Myristoylated alanine-rich C-kinase substrate - (Rat) |
| T17138 | CL1AA protein - rat |
| Q9JJZ7 | CTL1 protein.- Rattus norvegicus - (Rat). |
| S12002 | thyroid nuclear factor 1 - rat |
| A45988 | dentin matrix acidic phosphoprotein AG1 - rat |
| O88882 | Brain-enriched guanylate kinase-associated protein 2 - rat |
| Q99PJ6 | Zinc finger protein HIT-40 - Rattus norvegicus - (Rat). |
| Q7TQ14 | Aa1017.- Rattus norvegicus (Rat) . |
| I52644 | neuronal protein - rat |
| S58279 | GTP-binding protein rab4b - rat |

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| ADDB RAT | Beta adducin (Erythrocyte adducin beta subunit) - (Rat) |
| S03116 | gene 33 protein, hepatic - rat |
| O70596 | Kir2.4 protein.- Rattus norvegicus - (Rat). |
| JC8009 | choline dehydrogenase (EC 1.1.99.1) precursor - rat |
| Q64183 | Follicle-stimulating hormone receptor.- Rattus ap. |
| O88461 | VIP-receptor transcriptional repressor protein - (Rat). |
| Q9WU70 | B-tomosyn isoform.- Rattus norvegicus - (Rat). |
| I69008 | MHC class I RT1.E - rat |
| JQ1484 | activin receptor precursor - rat |
| S00726 | protein kinase A-raf-1 (EC 2.7.1.-) - rat |
| GCR RAT | Glucocorticoid receptor (GR) - (Rat). |
| I84639 | L-lactate dehydrogenase chain X [validated] - rat |
| HCK RAT | Tyrosine-protein kinase HCK (EC 2.7.1.112) - Rat |
| I59350 | karyopherin beta - rat |
| Q99MK3 | FE65.- Rattus norvegicus - (Rat). |
| AAH61804 | BC061884 NID: - Rattus norvegicus |
| JH0467 | fumarylacetoacetase (EC 3.7.1.2) - rat |
| CAC29904 | Sequence 3 from Patent EP1074627 (Fragment) - (Rat). |
| Q99P63 | SMHS2.- Rattus norvegicus - (Rat). |
| AAA41239 | RATGLUKB NID: - Rattus norvegicus |
| A54155 | natriuretic peptide receptor C precursor - rat |
| Q9ERC0 | Neuropeptide Y/peptide YY-Y2 receptor. - (Rat). |
| Q9WVK3 | Putative short-chain dehydrogenese/reductase mRNA, complete CDS - (Rat) |

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| T10756 | Nel-homolog protein - rat |
| I56579 | protein-tyrosine kinase (EC 2.7.1.112) batk - rat |
| B40204 | Na+/H+ exchanging protein 3 - rat |
| NOS3 RAT | Nitric-oxide synthase, endothelial (EC 1.14.13.39) |
| JC7668 | dipeptidyl-peptidase II (EC 3.4.14.2) - rat |
| A46631 | lactose-binding lectin L-36 - rat |
| Q99N78 | Transient receptor potential ca2+ channel 6A. - (Rat). |
| NAB1 RAT | NGFI-A binding protein 1 (EGR-1 binding protein 1) - rat |
| S15892 | pyruvate dehydrogenase (lipoamide) (EC 1.2.4.1) beta chain - rat |
| Q96J19 | Similar to adaptor-related protein complex 2, beta 1 subunit (Human) |
| A34366 | Ca2+/calmodulin-dependent protein kinase II delta chain - rat |
| Q9JHK1 | Caspase-9 (Caspase-9 long isoform). - (Rat). |
| Q8CJC4 | Frizzled-3 protein.- Rattus norvegicus - (Rat) . |
| Q80Z32 | Origin recognition complex subunit 1. - Rattus (Rat). |
| Q923L9 | Brain-muscle-ARNT-like protein 2b (Fragment). - (Rat). |
| T14272 | cortactin-binding protein 1 - rat |
| Q99NG8 | T:G mismatch thymine glycosylase. - (Rat) . |
| JC8027 | type 1 angiotensin II receptor associated protein 1 - rat |
| QRRTLD | LDL receptor precursor - rat |
| KIRTC | protein kinase C (EC 2.7.1.-) alpha - rat |
| I56526 | interleukin 1 receptor type I - rat |
| Q7TPJ9 | Ac2-141.- Rattus norvegicus - (Rat). |
| Q99MB4 | Dopamine responsive protein. - (Rat). |

| ATPB RAT | ATP synthase beta chain, mitochondrial precursor - (Rat). |
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| B43404 | beta-arrestin1 - rat |
| T14152 | synaptic scaffolding protein S-SCAM - rat |
| A34845 | protein-tyrosine-phosphatase, nonreceptor type 1B - rat |
| I53273 | gastric inhibitory polypeptide receptor - rat |
| Q8K583 | Fibronigen-like protein - Rattus norvegicus - (Rat). |
| JN0773 | calponin H1 - rat |
| A33270 | snRNF protein N - human |
| Q9Z220 | cp431 (Fragment).- Rattus norvegicus (Ratt). |
| KCRTS2 | stromelysin 2 (EC 3.4.24.22) precursor - rat |
| Q9JI01 | Corneal wound healing related protein. - (Rat). |
| Q7TP45 | Ab2-389 - Rattus norvegicus - (Rat). |
| A42345 | 3-hydroxybutyrate dehydrogenase precursor - rat |
| O70253 | Muscle carnitine palmitoyltransfarase I.- (Rat). |
| ADDA RAT | Alpha adducin (Erythrocyte adducin alpha subunit) - Rat |
| AAD50899 | Paired Ig-like receptor-B.- Rattus norvegicus - (Rat). |
| S49015 | receptor tyrosine kinase Ehk-1 - rat |
| A48319 | choline O-acetyltransferase (EC 2.3.1.6) - rat |
| CAD28393 | Sequence 10 from Patent WO0212341 - (Rat). |
| TXN2 RAT | Thioredoxin reductase 2, mitochondrial precursor - (Rat) |
| S44204 | procollagen-proline dioxygenase (EC 1.14 11.2) alpha chain - rat |
| T00011 | ccal protein - rat |
| Q9QYG9 | DLG6 gamma,- Rattus norvegicus - (Rat). |
| Q8CFC1 | Mina53.- Rattus norvegicus - (Rat). |

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| CAE84022 | BX883047 NID: - Rattus norvegicus |
| A55887 | caldesmon, non-muscle - rat |
| S11735 | resistance protein Mxl, interferon-regulated - rat |
| Q63774 | Hypothetical protein.- Rattus norvegicus - (Rat). |
| S00550 | Alzheimer's disease amyloid beta protein precursor - rat |
| T31425 | C-terminal domain-binding protein rA4, eplice form 2 - rat |
| S71321 | acyl-CoA dehydrogenase short-branched chain precursor - rat |
| AMD3 RAT | AMP deaminase 3 (AMP deaminase isoform E) - (Rat). |
| A42895 | H+/K+ exchanging ATPase alpha chain, colon Specific - rat |
| BAA82517 | AB024400 NID: - Rattus norvegicus |
| S47489 | receptor tyrosine kinase - rat |
| KIRTC2 | protein kinase C (EC 2.7.1.-) beta-II - rat |
| I52646 | DNA binding protein - rat |
| Q91WX0 | Complement factor H-related protein. - (Rat). |
| Q8R421 | Kidney-type glutaminase GAC isoform (Fragment). - (Rat). |
| I58311 | HMG-box containing protein 1 - rat |
| S65756 | gamma-aminobutyric acid receptor rho-3 chain precursor |
| O4RTV3 | Vitamin D3 25-monooxygenase cytochrome P450 27 precursor, mitochondrial - rat |
| DESM RAT | Desmin.- Rattus norvegicus - (Rat). |
| Q9JII4 | Prolactin-like protein L. - Rattus norvegicus - (Rat) . |
| Q99PJ7 | Zinc finger protein HIT-39.- Rattus norvegicus - (Rat). |
| I56896 | gene gfi-2 protein - rat |
| S35637 | high mobility group 1 protein homolog - rat (fragment) |

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| O4RTMC | unspecific monooxygenese cytochrome P450 1A1, hepatic |
| JW0046 | estrogen receptor beta2 - rat |
| AAB47997 | RNUB7983 NID: - Rattus norvegicus |
| A24639 | Na+/K+ exchanging ATPase alpha -1 chain [validated] - rat |
| AAA421683 | RATNCPR6 NID: - Rattus norvegicus |
| FGRTGA | Fibrinogen gamma-A chain precursor - rat |
| Q923L8 | Brain-muscle-ARNT-like protein 2c (Fragment) - (Rat). |
| 1AR0A | nuclear transport factor 2 mutant E42K, chain A - rat |
| Q9WV25 | RNA-binding protein SiahBP (Fragment). - (Rat). |
| JH0159 | histone H1d - rat |
| XDH RAT | Xanthine dehydrogenase/oxidase |
| Q7TMB6 | Aa2-174 (Cc1-38) .- Rattus norvegicus - (Rat). |
| Q7TP68 | Ab2-002 - Rattus norvegicus - (Rat). |
| A53204 | ribosomal protein L13a, cytosolic [validated] - rat |
| AAP37037 | AY283175 NID: - Rattus norvegicus |
| Q7TME5 | Ac1-581 (Ab1-181) (Ab1-216).- Rattus norvegicus - (Rat). |
| AAC40042 | AF041246 NID: - Rattus norvegicus |
| S26299 | transcription initiation factor IIB - rat |
| S31416 | pyruvate dehydrogenase (lipoamide) E1-alpha-2 chain - rat |
| S28901 | glutamate transport protein - rat |
| P70534 | Transmembrane protein tMDC I precursor. - (Rat). |
| Q9ESM0 | Inositol hexakisphosphate kinase. - (Rat). |
| Q9QWT3 | Retinoic acid receptor alpha2 (Fragment). - (Rat). |
| Q99JC8 | Neurotrypsin.- Rattus norvegicus - (Rat). |

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| Q9JJ10 | Neuronal C-SRC tyrosine-specific protein kinase. - (Rat). |
| AAC33823 | AF018261 NID: - Rattus norvegicus |
| P70610 | Doc2B.- Rattus norvegicus - (Rat). |
| NR41 RAT | Nerve growth factor induced protein I |
| AAF26890 | T cell receptor V delta 6 (fragment)  .- (Rat) . |
| O70513 | Mama.- Rattus norvegicus - (Rat). |
| C1TC RAT | C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase) [includes: methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) |
| Q9QVN5 | NEUROFASCIN isoform Rattus Sp. |
| Q8R1R5 | MIC2L1.- Rattus norvegicus - (Rat). |
| S16319 | secretin receptor - rat |
| VYRTD | vitamin D-binding protein precursor - rat |
| Q91YG2 | Carboxylic ester hydrolase (EC 3.1.1.1)  - (Rat). |
| KCB1 RAT | Potassium voltage-gated channel subfamily B member 1 - (Rat). |
| A49664 | activin type I receptor - rat |
| Q9Z251 | Lin-7-Ba.- Rattus norvegicus -  (Rat). |
| I56552 | synapse-associated protein 97 - rat |
| A35484 | tryptophan 2,3-dioxygenase (EC 1 13.11.11) - rat |
| S04328 | protein-tyrosine kinase (EC 2.7.1.112) flk - rat |
| A45445 | janusin precursor, long form - rat |
| HHRTGB | dnak-type molecular chaperone precursor - rat |
| Q924V4 | Peptide histidine transporter 1 homolog rPHT2. - (Rat). |
| A35816 | transcription regulator Pan-2 - rat |

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| O70260 | Potassium channel regulatory protein KChAP. - (Rat). |
| S45116 | natriuretic peptide clearance receptor homolog - rat |
| A31342 | fructose-bisphosphatase (EC 3.1.3.11), hepatic - rat |
| A41785 | system b(0,+) amino acid transporter-involved protein - rat |
| OQRT | hemopexin precursor - rat |
| Q31256 | MHC class I RT1.Au heavy chain precursor. - (Rat). |
| A46195 | cholecystokinin B receptor subtype - rat |
| Q9ESE8 | Inhibitor of apoptosis protein 2. - (Rat). |
| Q80YN6 | Ribonuclease/angiogenin inhibitor (Fragment). - (Rat). |
| S72266 | translation initiation factor eIF2B gamma chain - rat |
| A35362 | glycogen (starch) synthase (EC 2.4.1.11) , hepatic - rat |
| BAB63256 | AB054539 NID: - Rattus norvegicus |
| Q7TP63 | Ab2-064 - Rattus norvegicus - (Rat). |
| S21091 | dodecenoyl-CoA Delta-isomerase (EC 5.3.3.8) - rat |
| T31100 | probable potassium channel 2 - rat |
| Q7TNL4 | Ser/Thr-like protein kinase lyk4. - (Rat). |
| AAA21252 | RNU10097 NID: - Rattus norvegicus |
| AAQ96230 | AY383672 NID: - Rattus norvegicus |
| AAF26903 | T cell receptor J delta 2 (fragment). - (Rat). |
| A35447 | carnitine O-palmitoyltransferase II precursor, mitochondrial - rat |
| Q35217 | Hepatic multiple inosltol polyphosphate phosphatase - (Rat). |
| O88869 | PAM COOH-terminal interactor protein 1.- (Rat). |
| Q99M88 | Caspase-9 CDT isoform - Rattus norvegicus - (Rat). |
| Q91ZL9 | Transient receptor potential channel 4 beta-2 splice variant - (Rat). |

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|---|---|
| JC7782 | C1- pump- associated 55k protein, CIP55 - rat |
| JC4386 | adenylyl cyclase-associated protein CAP2 - rat |
| JH0674 | L-proline transport protein - rat |
| Q921A2 | Proton myo-inositol transporter. - (Rat). |
| AMPB RAT | Aminopeptidase B (EC 3.4.11.6) (Ap-B) |
| CAB70590 | Sequence 3 from Patent WO9909054 - Rattua rattus |
| Q8R5M4 | FIP2 like protein.- Rattus norvegicus - (Rat). |
| Q9Z0N8 | ZG29p.- Rattus norvegicus - (Rat). |
| B53764 | beta2-chimerin, cerebellar - rat (fragment) |
| Q7TSS5 | CD2-associated protein. - (Rat). |
| WHRTW | tryptophan 5-monooxygenase (EC 1.14.16.4) - rat |
| G42148 | GTP-binding protein rab16 - rat |
| S21126 | proteasome endopeptidase complex chain RC1 - rat |
| Q9JI12 | Differentiation-associated Na-dependant inorganic phosphate cotransporter. - (Rat) |
| S52284 | Lumicon, secretory intersticial proteoglycan precursor - rat |
| 1B9XC | phosducin mutant YES, chain C - rat (fragments) |
| Q920G0 | Putative SKAP55.- Rattus norvegicus - (Rat). |
| Q9JHY1 | Junctional adhesion molecule JAM. - (Rat). |
| KIRTCE | protein kinase C (EC 2.7.1.-) epsilon - rat |
| JE0155 | mitochondrial inner membrane translocating protein, rTIM44 - rat |
| Q91XV2 | Gonadotropin inducible ovarian transcription factor 1- rat |
| G6PD RAT | Glucose-6-phosphate 1-dehydrogenase (G6PD) - (Rat). |
| Q8K4H4 | FHA-HIT.- Rattus norvegicus - (Rat). |

| | |
|---|---|
| I2C2 RAT | Eukaryotic translation initiation factor 2C 2 (eIF2C 2) |
| NCT3 RAT | Natural cytotoxicity triggering receptor 3 precursor |
| Q9TP50 | MHC class Ia antigen (Fragment). - (Rat). |
| O54963 | Zinc finger transcription factor REST protein. - (Rat). |
| CAA40861 | RNA2C4 NID: - Rattus norvegicus |
| Q9ESY1 | TEMO.- Rattus norvegicus - (Rat). |
| CAC33148 | Sequence 51 from Patent WO0100826. - Rattus rattus |
| Q9QVT8 | BSPL=NEURAL membrane CD26 peptidase-like protein.- Rattus sp. |
| S52477 | disintegrin (EC 3.4.24.-) precursor - rat (fragment) |
| AAG59704 | TCR delta (Fragment).- (Rat). |
| Q9QYC2 | RRM-type RNA-binding protein BRPTB (PTB-like protein) (Splicing regulator nPTB2) - Mus musculus (mouse) and Rattus rattus |
| Q9R186 | Calcium transporter CaT1.- Rattus norvegicus - (Rat). |

### 4.3.2 IPA results

4.3.2.1 Normal and hydrocephalic CSF do not share the same protein profile and metabolic pathways

Core IPA analysis produced two large graphs representing normal and abnormal CSF respectively. The left axis on the graphs relates to the $-$ log of p-values (significance of results) meaning that the association made by IPA between the selected proteins found in CSF and the chosen canonical pathways did not happen by chance. Therefore, the smaller the p value, the greater the chance the IPA association was not matched randomly. In other words, the smaller the p value the greater the value of the "-log (P-value)". On the other hand, the right axis indicates the ratio. The ratio is the number of proteins found in the experimental dataset that fall in a canonical pathway divided by the total number of proteins in the reference canonical pathway. Ratio values below the threshold ratio should not be considered. "-log (P-value)" and ratio should increase together, otherwise the obtained values may represent only false positives (www.ingenuity.force.com).

In the present study, grey color bars were obtained, indicating that this analysis does not have directionality (i.e. we don't know whether gene expression is upregulated or downregulated), as we did not include data about quantification of gene expression. Moreover, grey bars mean that the set of molecules included in the canonical pathways, although eligible for this pathway, still lack a predictive value. Figures 4.3 and 4.4 shows canonical pathways found for category 1 (proteins only found in normal CSF) and category 2 (proteins only found in abnormal CSF). Canonical pathways with ratios above the threshold are highlighted with red arrows on Figure 4.3, and purple arrows on Figure 4.4 below.

**Figure 4.3. Canonical pathways for proteins only found in normal CSF.**

287

Extrinsic Prothrombin Activation Pathway · Mitochondrial L-carnitine Shuttle Pathway · Cholecystokinin/Gastrin-mediated Signaling · Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis · Wnt/Ca+ pathway · Gas Signaling · Wnt/β-catenin Signaling · Polyamine Regulation in Colon Cancer · Endothelin-1 Signaling · Colorectal Cancer Metastasis Signaling · PCP pathway · Glutamine Degradation I · GABA Receptor Signaling · RhoA Signaling · Regulation of the Epithelial-Mesenchymal Transition Pathway · Cell Cycle Control of Chromosomal Replication · PEDF Signaling · Chemokine Signaling

**Glutaminase (GLS)**

288

FXR/RXR Activation

Basal Cell Carcinoma Signaling

STAT3 Pathway

GNRH Signaling

Intrinsic Prothrombin Activation Pathway

Leukocyte Extravasation Signaling

Human Embryonic Stem Cell Pluripotency

Relaxin Signaling

Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza

Aryl Hydrocarbon Receptor Signaling

Uracil Degradation II (Reductive)

Thymine Degradation

Geranylgeranyldiphosphate Biosynthesis

TWEAK Signaling

Melanocyte Development and Pigmentation Signaling

Coagulation System

Glioblastoma Multiforme Signaling

UVA-Induced MAPK Signaling

**Dihydropyrimidine dehydrogenase (DPYD)**

**Farnesyldiphosphate synthase (FDPS)**

289

**Figure 4. 4. Canonical pathways for proteins only found in abnormal CSF.**
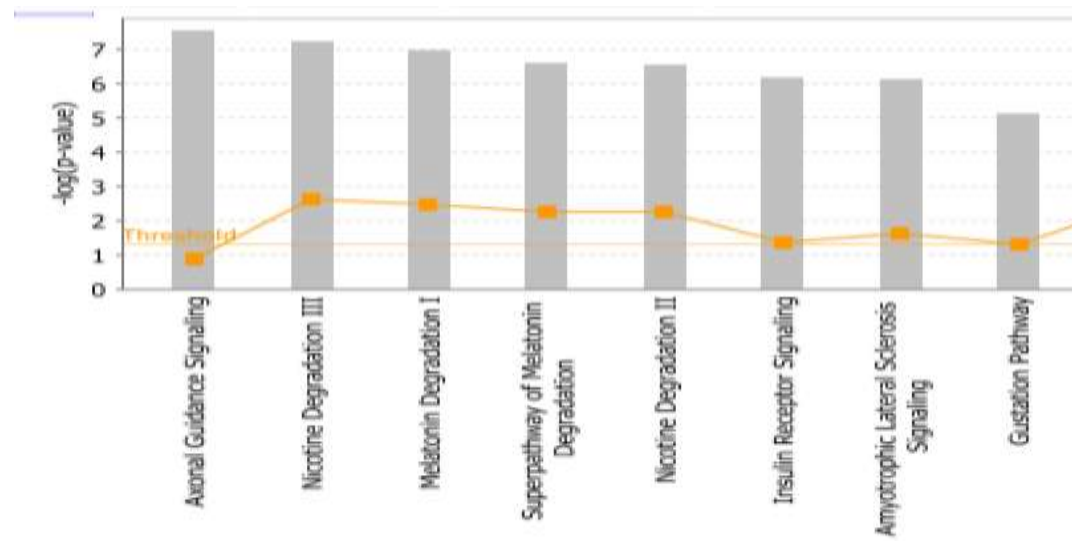
Tec Kinase Signaling

Reelin Signaling in Neurons

Wnt/Ca+ pathway

LPS/IL-1 Mediated Inhibition of RXR Function

D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)

D-myo-inositol (1,3,4)-trisphosphate Biosynthesis

Human Embryonic Stem Cell Pluripotency

fMLP Signaling in Neutrophils

Serotonin Degradation

Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency

Superpathway of Serine and Glycine Biosynthesis I

Synaptic Long Term Depression

GM-CSF Signaling

Endothelin-1 Signaling

D-myo-inositol-5-phosphate Metabolism

UVA-Induced MAPK Signaling

RhoGDI Signaling

FcγRIIB Signaling in B Lymphocytes
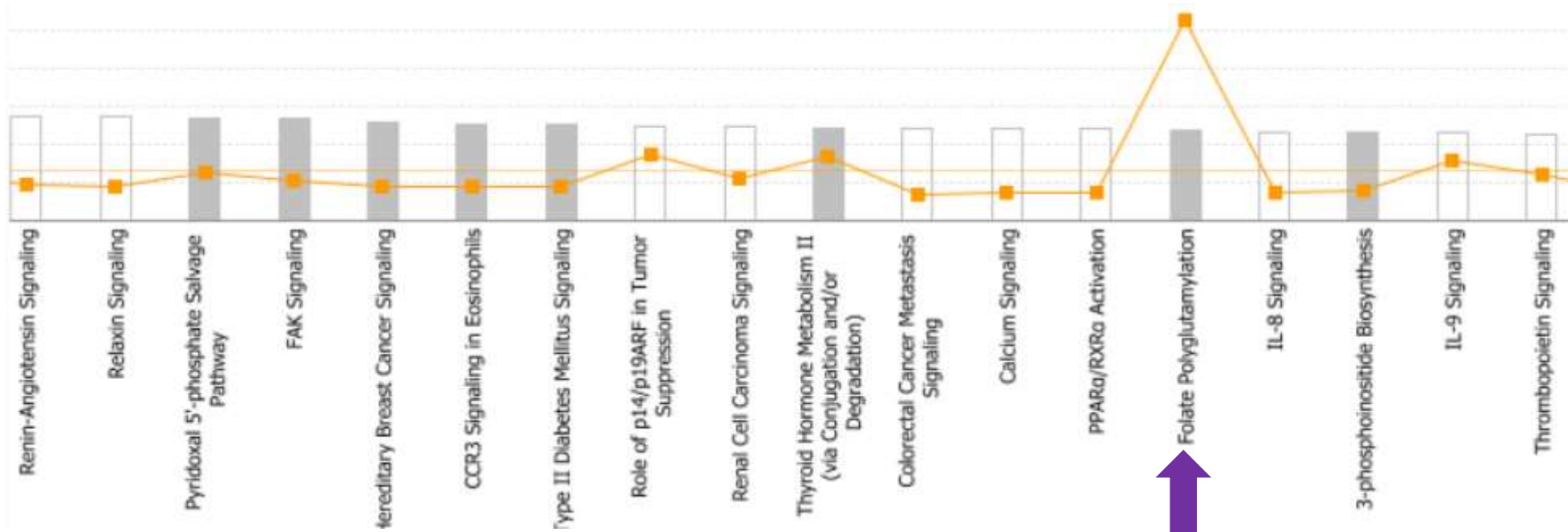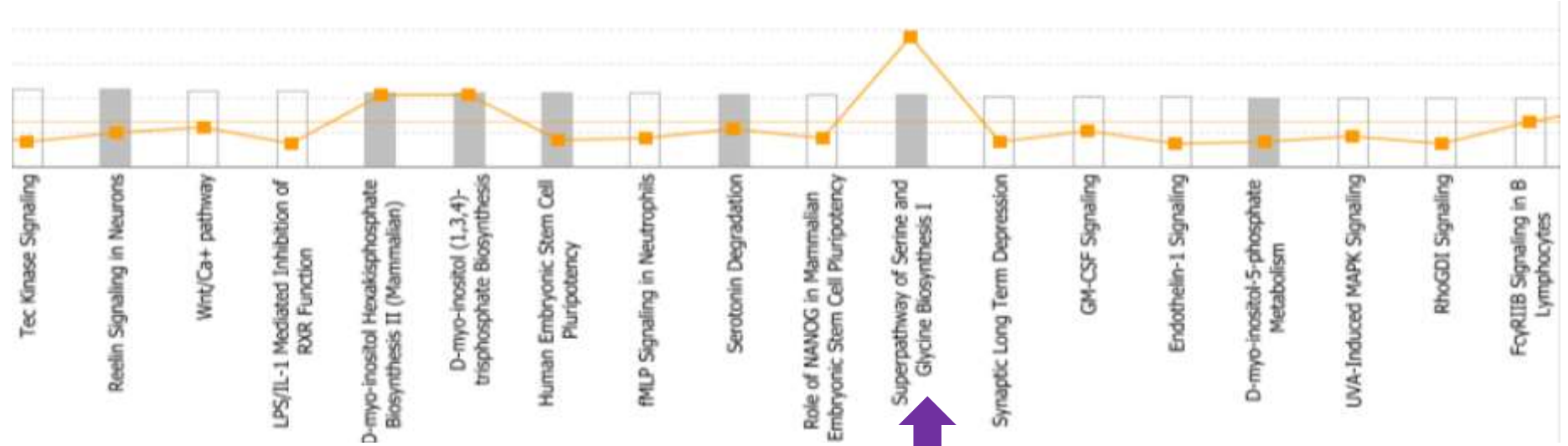
**SERINE HYDROXYMETHYL TRANSFERASE (SHMT1)**

**PHOSPHOHYDROXYTHREOINE AMINOTRANSFERASE (PSAT1)**

293

Calcium Transport I
Folate Transformations I
MSP-RON Signaling Pathway
IGF-1 Signaling
IL-17 Signaling
Ephrin B Signaling
D-myo-Inositol (1,4,5)-Trisphosphate Biosynthesis
Dopamine-DARPP32 Feedback in cAMP Signaling
Ovarian Cancer Signaling
HIF1α Signaling
Paxillin Signaling
HER-2 Signaling in Breast Cancer
IL-4 Signaling
PDGF Signaling
HGF Signaling
Leukocyte Extravasation Signaling
CNTF Signaling

ATP2A2
ATP2C1
ATP2C1
CALCIUM
PUMPS

MTHFD1
SHMT1

DNA Double-Strand Break Repair by Homologous Recombination

NRF2-mediated Oxidative Stress Response

Production of Nitric Oxide and Reactive Oxygen Species in Macrophages

CTLA4 Signaling in Cytotoxic T Lymphocytes

RANK Signaling in Osteoclasts

CD28 Signaling in T Helper Cells

P2Y Purigenic Receptor Signaling Pathway

PAK Signaling

Regulation of the Epithelial-Mesenchymal Transition Pathway

γ-glutamyl Cycle

Clathrin-mediated Endocytosis Signaling

Glycine Biosynthesis I

Factors Promoting Cardiogenesis in Vertebrates

PI3K/AKT Signaling

Mismatch Repair in Eukaryotes

IL-17A Signaling in Airway Cells

Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes

SHMT1
PSAT1

4.3.2.2 <u>Different top canonical pathways were found in normal and hydrocephalic CSF</u>

IPA analysis gave rise to graphs representing all canonical pathways where the molecules found in CSF may fall. Since only data with values higher than the threshold (top canonical pathways) can be considered to have any significance, we have grouped these metabolic pathways according to ratio and –log (p-value) as shown in tables 4.3 (Normal CSF) and 4.4 (Abnormal CSF) :

| CANONICAL PATHWAY | MOLECULES | RATIO | -LOG(P-VALUE) |
|---|---|---|---|
| Glutamine degradation I | GLS | 0.4 | >1.5 |
| Uracil degradation II | DPYD | 0.2 | 1.09 |
| Thymine degradation | DPYD | 0.2 | 1.09 |
| Geranylgeranyl diphosphate biosynthesis | FDPS | 0.2 | 1.09 |

**Table 4.3. Top canonical pathways uniquely identified in normal CSF(ratio>threshold)**

Table 4.3 shows how normal CSF contains molecules that fall mainly in the glutamine degradation I metabolic pathway (glutaminase, GLS). This top pathway presented the highest molecule ratio (0.4) and significance at $p<0.05$; ($p=0.03$). Interestingly, uracil and thymine degradation pathways were also selected by IPA, as well as geranylgeranyl diphosphate biosynthesis, but they had half the ratio values of the top pathway (glutamine degradation), showing p values $> 0.05$ and therefore indicating no significance.  This was due to a low –log (p value); (>1) as p value $=0.08$; -log (0.08) $=1.09$. Nevertheless, IPA identified the enzyme, dihydropyrimidine dehydrogenase (DPYD), which falls in both uracil and thymine degradation pathways. Similarly, detection of the enzyme farnesyl diphosphate synthase (FDPS) was also made by IPA and associated with biosynthesis of geranylgeranyl diphosphate. All molecules found in normal CSF by IPA are listed in Appendix 1.

| CANONICAL PATHWAY | MOLECULES | RATIO | -LOG(P-VALUE) |
|---|---|---|---|
| Folate polyglutamation | MTHFD1 SHMT1 | 0.5 | >2 |
| Superpathway of serine & glycine biosynthesis I | SHMT1 PSAT1 | 0.3 | >2 |
| Calcium transport I | ATP2A2 ATP2C1 ATP2C2 | 0.3 | >3 |
| Folate transformations I | MTHFD1 SHMT1 | 0.3 | >3 |

**Table 4.4 Top canonical pathways uniquely identified in abnormal CSF(ratio>threshold).**

With regard to top canonical pathways identified in abnormal CSF, IPA found 50% of molecules pertaining to the folate polyglutamation pathway (Table 4.4). These molecules were methyltetrahydrofolate dehydrogenase 1 (MTHFD1) and serine hydroxymethyltransferase (SHMT1) (ratio=0.5). This value concurred with the association's significance made by IPA, which showed –log (p value) >2 and, therefore, significant at p<0.01.

IPA analysis also revealed other three important metabolic pathways, each with 30% of their related molecules present in abnormal CSF. These molecules belonged to the superpathway of serine and glycine biosynthesis I: SHMT1 and phosphohydroxythreonine aminotransferase 1 (PSAT1), calcium transport I: ATP2A2, ATPC1 and ATPC2 calcium pumps) and folate transformations I: MTHFD1 and SHMT1, respectively. Ratio values (0.3) for these three pathways were all in agreement with IPA associations' significance values (p< 0.01), implying a significant relationship between molecules detected in abnormal CSF and the IPA selected metabolic pathway.

All molecules found in abnormal CSF by IPA are listed in Appendix 2.

4.3.2.3 Different biological functions were associated to normal and hydrocephalic CSF

IPA analysis also allows matching of biological functions/dysfunctions with sets of proteins/genes identified in CSF. Our hypothesis is that the presence of specific metabolically related proteins in CSF is indicative of a given biological process occurring in this fluid. The obtained p values were all <0.05, and therefore the associations between molecules and biological function should be considered significant. A summary of the top biological functions detected in normal and abnormal CSF is described in tables 4.5 and 4.6 below.

| BIOLOGICAL FUNCTION | % PROTEINS IN NORMAL CSF LINKED TO SPECIFIC BIOLOGICAL FUNCTION |
|---|---|
| Cellular growth and proliferation | 17.93 |
| Tissue development | 16.28 |
| Cell morphology | 15.05 |
| Cellular function and maintenance | 14.22 |
| Nervous system development and function | 13.19 |
| Organismal development | 13.19 |
| Molecular transport | 12.98 |
| Cellular assembly and organization | 11.54 |
| Embryonic development | 11.34 |
| Behaviour | 6.39 |

**Table 4.5. IPA summary of biological functions associated with proteins found in normal CSF.**

Percentages were calculated taking into account the overall number of proteins considered in the IPA study for normal and abnormal CSF respectively. Overall, table 7 shows that most of the biological functions identified by IPA in normal CSF related to cell development, growth, and assembly and organization, with proteins within a 10-20% range falling in those biological processes. Nevertheless, proteins linked to molecular transport (12.98%) were also detected, as were behavioural proteins (6.39%). Molecules corresponding to biological functions identified by IPA in normal CSF are listed in Appendix III.

| BIOLOGICAL FUNCTION | % PROTEINS IN ABNORMAL CSF LINKED TO SPECIFIC BIOLOGICAL FUNCTION/DYSFUNCTION |
|---|---|
| Liver hyperplaxia/hyperproliferation | 39 |
| Cellular function and maintenance | 34.71 |
| Molecular transport | 33.88 |
| Cell death and survival | 33.67 |
| Organismal development | 31.81 |
| Cell morphology | 31.40 |
| Cell assembly and organization | 26.03 |
| Nervous system development | 25.20 |

**Table 4.6. IPA summary of biological functions associated with proteins found in abnormal CSF.**

With respect to biological functions linked to proteins found uniquely in abnormal CSF, IPA analysis found that more than 30% of molecules present in this CSF were associated with liver hyperproliferation, cellular function and maintenance, and cell death and survival (table 4.6). Abnormal CSF also shared biological functions with normal CSF, and molecular transport, organismal development cell morphology and assembly, as well as nervous system development were common to both types. However, the percentage of proteins related to those biological functions was higher in abnormal CSF >30% than in normal CSF (<18%), (table 4.5 and table 4.6). To understand the high percentage values in table 4.6, it is necessary to also understand that one protein can be shared by more than one canonical pathway. Molecules corresponding to biological functions identified by IPA in abnormal CSF are listed in Appendix IV.

## 4.4 CONCLUSION-DISCUSSION

CSF is a protein's reservoir which reflects the physiological state of the brain. Thus, changes in normal CSF constituents, and variations in the normal protein profile specifically can be used for disease prediction (Puchades et al. 2003) and clinical diagnoses by means of clinical tests based on disease biomarker identification (Anderson, 2002).

Since LCMS and IPA aims to identify and quantify molecules that are present in the same chemical class and/or metabolic pathway (Metz et al. 2007), we propose that the specific proteins exclusively present in hydrocephalic CSF should be considered biological markers of congenital hydrocephalus. Even more importantly, we demonstrate that these molecules are related and fall into relevant canonical pathways identified by IPA analysis. Consequently, we believe our results can be useful as a starting point to develop a clinical tool for diagnosis of congenital hydrocephalus and future adequate choice of treatment.

In past studies, LCMS CSF analysis of normal and hydrocephalic fetal CSF produced two corresponding data sets that were compared with the aim of finding differences in folate enzyme profile. Only one folate enzyme was found not to be present in hydrocephalic CSF i.e.: FDH, and for this reason, most of the latest studies in our lab were related to the study of this folate binding protein mainly.

In the present study, we pay special attention to the identification of metabolic pathways potentially activated and inactivated in normal and abnormal brain, based on the presence and absence of proteins in each specific type of CSF. The strategy consists of grouping each LCMS data-set, representing normal and abnormal CSF respectively into two groups: a first group including proteins which are not common to normal CSF and present only in

abnormal CSF, and a second group containing proteins which are not common to abnormal CSF but present in normal CSF. Excel analysis was used to group the data in this fashion. Once proteins have been appropriately grouped, IPA analysis was carried out for identification of metabolic pathways and biological functions unique to each type of CSF. Interestingly, IPA analysis of normal CSF allowed the detection of proteins (GLS) present in a key metabolic pathway, the glutamine degradation pathway. IPA association between CSF proteins and this canonical pathway was significative ($p<0.05$), as were the associations also created by IPA between molecules in  hydrocephalic CSF and folate polyglutamation (MTHFD1 and SHMT1), the superpathway of serine and glycine biosynthesis I (PSAT1 and SHMT1), calcium transport I (ATP2C2, ATP2C1, ATP2A2) and folate transformations I pathways (MTHFD1 and SHMT1). Interestingly, SHMT1 was an enzyme present in most of the metabolic pathways associated with hydrocephalic CSF, which highlights this protein as a key molecule involved in the outcome of congenital hydrocephalus.

Given our approach in this study, and according to our results, we postulate that glutamine degradation is activated in normal CSF, but inactivated in abnormal CSF. A lack of glutamine degradation by the enzyme GLS implies that glutamine-degradation associated biological processes are impaired in hydrocephalus, with negative consequences for brain development. Furthermore, glutamine degradation is needed  for the net production of both purines and pyrimidines for nucleotide biosynthesis and cell division, as well as for synthesis of precursors for N-linked and O-linked glycolsylation reactions, for modifications of lipids and proteins involved  in signal transduction, protein transport and other processes (DeBerardinis et al. 2007). Moreover, glutamine is converted into

glutamate by GLS in axonal terminals of neurons in the presence of water, producing ammonia (Botman et al. 2014) as below:

$$\text{Glutamine} + H_2O \xrightarrow{\text{GLS}} \text{Glutamate} + NH_3$$

Glutamate is the most abundant neurotransmitter in the nervous system that is involved in neural activation and synaptogenesis. It is also crucial for the production of glutamate pools for polyglutamation of intracellular folate and folate storage (Ramaekers et al. 2004). Glutamate pools are also needed for cells to acquire cysteine, and for the production of glutathione, an anti-oxidant needed for cell survival in situations of high cell activity and oxidative stress (Estrela et al. 2006). Finally, glutamine has a role in extracellular signal-regulated protein kinase signalling (ERK), through its conversion to glutamate and the binding of this neurotransmitter to receptors expressed in neuronal tissues. This indicates that glutamate also has a role in signalling and activation of ERK and phosphatidylinositol 3'kinase signalling (Skerry and Genever 2001, Nicoletti et al. 2007). Thus, this study's outcome reveals GLS as an important candidate for future genetic expression analysis to investigate whether or not GLS is downregulated in congenital hydrocephalus as it is not found by IPA in hydrocephalic CSF but in normal CSF.

In conclusion, since glutamine degradation is crucial for supporting cell growth, proliferation and survival, it is not surprising that the glutamine degradation pathway is triggered in the normal brain during development, and that molecules linked to this pathway were found by IPA analysis in normal CSF. Also, because these molecules were not present in abnormal CSF, we suggest that the hydrocephalic brain lacks the ability to produce glutamate from glutamine via the normal route through GLS. Consequently the functions attributed to GLS are bound to be negatively affected.

With regards to our results from hydrocephalic CSF, these are controversial as folate polyglutamation seems to be activated. This result suggests that a source of glutamate other than from the GLS route is available in hydrocephalus. This may be explained by the existence of alternative metabolic routes for glutamate production in the body. Thus, glutamate is produced in the GABA shunt cycle from α-ketoglutarate or 2-oxoglutarate by glutamate dehydrogenase (GLDH) in parallel with the production of semialdehyde succinic acid (SSA) from γ-aminobutyric acid GABA (synapsis inhibitory neurotransmitter) by GABA transaminase, as shown below:

α-ketoglutarate ⟶ Glutamate

SSA        GABA

Interestingly, SSA is toxic to cells if it is not converted to succinic acid by the enzyme succinic semialdehyde dehydrogenase (SSADHD). Deficiencies in this enzyme are linked to increased gaba-hydroxybutirate (GHB), a neurotoxic agent that accumulates in CSF and leads to developmental delay and mental retardation (Robbins E. 2004).

However, GABA receptor GABR2 (appendix 2) rather than GLDH and SSADH was identified by IPA suggesting GABA signalling metabolic pathway is activated. Yet, this pathway was lower than the threshold chosen for IPA analysis (0.1). IPA low ratio and/or underdetection of molecules like GLDH and SSADH is common in LCMS analysis due to undersampling, a drawback of this technology that leads to molecules being undetected (Metz et al. 2007). Therefore, we propose GLDH and SSADH to be part of future gene expression studies to verify if they are involved in congenital hydrocephalus at all.

Moreover, there is an alternate route for production of glutamate from α-ketoglutarate or 2-oxoglutarate, as source of glutamate which is needed for folate polyglutamation. Folates are bound to just one molecule of glutamate in CSF (monoglutamated state), and gain access to choroidal cells through active transport mediated by folate receptors. Once monoglutamated folates are inside cells, they are converted into folyl-polyglutamated folates by folylpoly-gamma-glutamate synthetase for storage, or they are transformed into dihydrofolate and tetrahydrofolate in the folate metabolic cycle (Ramaekers et al. 2004). In this context, activation of folate polyglutamation in hydrocephalic CSF means an elevated use of glutamate, and we suggest this is provided by tricarboxylic cycle activation in mitochondria. Interestingly, the Krebs cycle is intertwined with the glycine and serine superpathway another metabolic pathway identified by IPA through detection of PSAT1 and SHMT1. α-ketoglutarate or 2-oxoglutarate is produced in the glycine and serine superpathway, thereby feeding the tricarboxylic cycle with this metabolite. This concurs with our results; as more than 30% of molecules (PSAT1 and SHMT1) in abnormal CSF belonged to the glycine and serine superpathway. Therefore, this finding suggests that the glycine and serine metabolic pathways may be activated in congenital hydrocephalus; they are likely to feed the Krebs cycle for production of α-ketoglutarate or 2-oxoglutarate, and later of glutamate for folate polyglutamation.

Intracellular calcium is needed for mitochondria (Krebs cycle) and reticulum endoplasmic activity in neurons, and has a key role as a second messenger in glutamate release (Miller 1991). This means that glutamate release depends on activation of calcium flux through internal membranes. On the other hand, membrane calcium transport is dependent on receptor activation by glycine and glutamate (Laube et al. 1997 and Li et al. 2009). These facts are also directly connected to our results regarding stimulation of calcium transport

metabolic pathway and serine and glycine biosynthesis superpathway, linked to the ATPase pumps (ATP2C2, ATP2C1, and ATP2A2) found in hydrocephalic CSF by IPA analysis. In conclusion, IPA identification of the described metabolic pathways in abnormal CSF suggests that calcium transport (calcium flux trough membranes) followed by glutamate synthesis activation and glycine production as well as α-ketoglutarate or 2-oxoglutarate synthesis in the glycine and serine biosynthesis superpathw are all activated and linked in congenital hydrocephalus.

A-ketoglutarate or 2-oxoglutarate produced in the glycine and serine biosynthesis superpathway is converted to glutamate for polyglutamation in the GABA shunt cycle (Rowley 2012). The glycine and serine biosynthesis superpathway and concomitant production of 2-oxoglutarate is briefly described below:

3-phospho-hydroxypyruvate → (3-phospho-serine aminotransferase; L-glutamate → 2-oxoglutarate) → 3-phospho-L-serine → L-serine

Glycine + 5,10-methylen-THF ← (5,10-methylentetrahydrofolate: glycine hydroxymethyltransferase / Serine hydroxymethyltransferase) → L-serine + THF
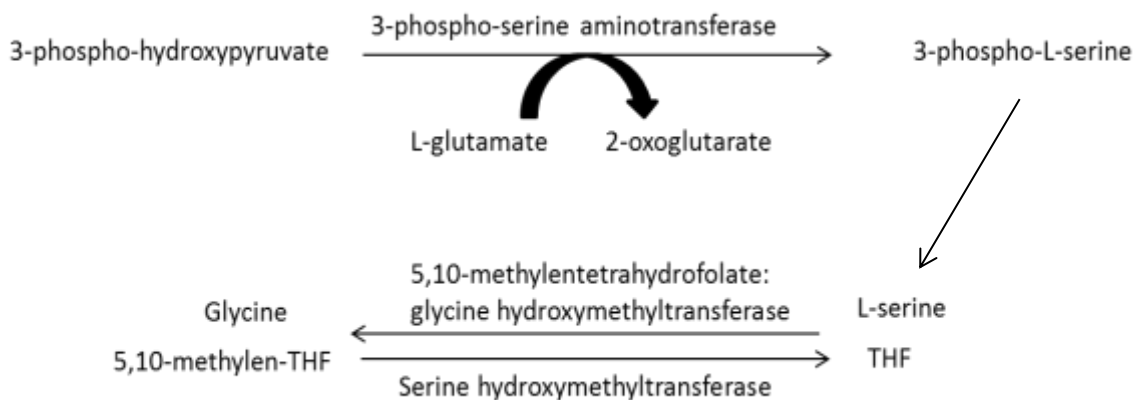
Furthermore, folate transformations I (folate metabolic reactions involved in production of THF) appear to be activated since MTHFD1 and SHMT1 were also identified by IPA in abnormal CSF. This outcome is inconclusive as these folate transformations are needed in normal brain, and should be present in normal CSF too (see diagram below). This result may imply folate transformations I pathway happens at a different time point in development, and for this reason they are not present in normal CSF at the embryonic day

chosen for our investigations, yet, present in hydrocephalic CSF. In any case, this finding agrees with the serine and glycine biosynthesis superpathway too and both pathways point to a production of THF as indicated in the glycine and serine biosynthesis superpathway described above. THF is a folate that seems to be in demand in the hydrocephalic brain, and it has been proven that treatment with this folate, when stable, improves congenital hydrocephalus and encourages growth of cerebral cortex cells. In view of this facts, we hypothesize that the glycine and serine biosynthesis metabolic superpathway and the folate transformations I canonical pathway may be activated to supply THF due to a lack of this folate in the hydrocephalic brain. However, THF transport and delivery to areas like the cerebral cortex may be compromised by the low FDH levels identified in hydrocephalic CSF (Cain et al. 2009), since FDH is thought to act as a THF reservoir and folate transporter. In conclusion, the folate enzymes MTHFD1, SHMT1 and FDH should be considered for future genetic expression analysis to establish if they are overexpressed or under-expressed as well as to explore their potential as biomarkers of this condition.

In respect of biological functions and dysfunctions associated with the CSF protein profile, the most relevant outcome was the presence of nearly 40% of proteins in hydrocephalic CSF falling into the liver hyperplaxia and proliferation dysfunction. This finding suggests that there is a degeneration of the fetal liver or the mother's liver, with maternal liver proteins reaching the CSF through the placenta. Furthermore, the liver expresses PCFT and FRα folate transporters, and provides plasma with soluble folate transporters (sFRα), hence; deterioration of liver in the mother means low levels of sFRα in plasma and folate provision to the brain through the placenta, as well as to CSF at early pregnancy (Wani et al. 2013) .

Interestingly, molecular transport proteins were found in both normal and abnormal CSF, and therefore this finding is inconclusive according to our experimental approach based on protein presence (gene activation) and non-presence (gene inactivation). This finding confirms the need for quantification of gene expression and its inclusion in the IPA analysis, and only comparison between IPA analyses, along with gene expression measurements in each type of CSF, will allow any conclusions about molecular transport. In spite of this result, this finding suggests that our studies are leading in the right direction, as FDH is a folate binding protein that is thought to be involved in vitamin transport in CSF, located together with FRα within exosomes in CSF. Finally, another biological function exclusively related to hydrocephalic CSF was cell death and survival, with more than 30% proteins belonging to this function. This function was not identified by IPA analysis in normal CSF, which is controversial as it is known that apoptosis and DNA degradation are part of normal brain development, and programmed cell death is important for the formation of the nervous system (Vaux et al. 1999). Therefore, questions arise about LCMS identification of small concentrations of proteins in normal CSF lower than 50 femtomols that may pass undetected. This conclusion also applies to the folate transformations I canonical pathway, explained previously, which is not identified in normal CSF either. Another possibility is that may these two processes happen later in development for normal CSF, and that they are activated specifically at embryonic day 18 only in hydrocephalic CSF.

Due to the limited time frame of this investigation, gene expression quantification was not performed. This was a drawback to our study, and the reason for the lack of a final predictive value in terms of gene activation/inactivation. Therefore, this study should be considered as a starting point to more extensive and complementary research in the future.

RTPCR assays should be performed to measure overexpression or underexpression of "focus genes" (GLS, MTHFD1, SHMT1, PSAT1, ATP2C2, ATP2C1, ATP2A2) in the canonical pathways elected by IPA followed by IPA re-analysis with integration of gene expression data. Nevertheless, only significant IPA associations ($p < 0.05$) between CSF proteins and top canonical pathways were studied. Thus, our research provides a useful source of reference data for future production of efficient and reliable diagnostic tests based on the identification of molecules unique to hydrocephalic CSF and/or decreased or increased mRNA quantification in relation to normal expression levels.

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# CHAPTER 5. CONCLUDING REMARKS, DISCUSSION, AND FUTURE AIMS

**5.1.1.** <u>Chapter 2 concluding remarks flow chart. Figure 5.1:</u>



LCMS ANALYSIS OF NORMAL AND HC CSF

**+ FDH IN NORMAL CSF**

**− FDH IN HC CSF**

**− FDH IN HC CSF SUGGESTS IMBALANCED FOLATE METABOLISM**

**FDH/FOLATES EFFECT ON CEREBRAL CORTEX CELL PROLIFERATION: "IN VITRO" STUDIES**

↑FDH & constant 5mTHF = ↓ CELL GROWTH

↑5 mTHF & constant FDH = ↓ CELL GROWTH

STABILIZED IgG-THF = ↑CELL GROWTH

POTENTIAL TREATMENT FOR CONGENITAL HYDROCEPHALUS?

DOES FDH BIND TO 5mTHF ? ELISA STUDIES SHOWS FDH AFFINITY FOR 5mTHF

HC: HYDROCEPHALIC

<u>FDH ROLE IN REGULATION OF 5mTHF LEVELS IN CSF</u>? OUR RESULTS AGREE WITH ANGUERA ET AL. STUDIES (2006)

<u>FDH ROLE IN BIOAVAILABILITY OF METHYL GROUPS THROUGH 5mTHF PROVISION?</u> If ↓ FDH = ↓ 5mTHF DELIVERY TO CELLS = ↓ METHYL GROUPS = ↓ METHIONINE = ↑HOMOCYSTEINE= HYPOMETHYLATION= ↓ BRAIN DEVELOPMENT

<u>FDH ROLE IN FOLATE TRANSPORT ?</u> IMMUNOHISTOCHEMISTRY SHOWS FDH CO-LOCALIZE WITH FRα IN EXOSOMES AND INTRACELULLARLY WITH ↓ FDH & ↑ FRα IN ABNORMAL BRAIN; IS THERE FRα OVEREXPRESSION TO COMPENSATE FOR ↓ FDH ?

<u>ARE THERE ALTERED FDH mRNA PROCESSING AND/OR PROTEIN POST-TRANSLATIONAL MODIFICATIONS IN HC BRAIN?</u> FDH GENE EXPRESSION ANALYSIS BY RTPCR SHOW NO CORRELATION WITH PROTEIN EXPRESSION

### 5.1.2. Chapter 2 concluding remarks and discussion

☐ POTENTIAL ROLE OF FDH IN REGULATION OF 5mTHF LEVELS IN CSF

"In vitro" work and "in house" immunoassay studies demonstrated that FDH has affinity for 5mTHF, suggesting a role of FDH in regulation of 5mTHF levels in CSF when FDH is present at high concentration $p < 0.001$. Therefore, we propose that FDH binds to folates acting as a 5mTHF reservoir in CSF when FDH is abundant. On the other hand, at low concentration, FDH detaches from 5mTHF and both molecules enter the cells, activating the folate metabolic cycle and encouraging cell growth. Thus, a total lack of FDH in CSF, i.e.: hydrocephalic CSF can be detrimental for regulation of extracellular and intracelullar 5mTHF, which may have a negative knock-on effect on areas of the brain such as the cerebral cortex and the arachnoid.

☐ POSSIBLE ROLE OF FDH IN 5mTHF HANDLING AND TRANSPORT ALONG WITH FRα.

FDH affinity studies using commercial and "in house" ELISAs, as well as tissue immunolocalization analysis, reveal FDH as a protein involved in 5mTHF handling. FDH may work in a similar fashion to FRα, a soluble membrane receptor with high affinity for 5mTHF. In the present work, FDH, FRα and 5mTHF share the same location within the choroid plexus, CSF exosomes and the cerebral cortex, which is evidence that the two proteins work together to provide the brain with folate.

- LOCATION OF FDH ALONG WITH FRα WITHIN EXOSOMES.

5mTHF is located with FDH and FRα within vesicles in CSF. 5mTHF transport within vesicles forming folate-protein conjugates suggests that this is a protective mechanism against the physicochemical changes between the extracellular media or CSF and within the cell's different compartments as folates are sensitive to oxygen and changes in pH.

- STABLIZED THF AS A SUITABLE FOLATE CANDIDATE FOR TREATMENT OF CONGENITAL HYDROCEPHALUS.

FDH's product of reaction (THF) encourages cell growth only if linked to complementary molecules such as THF antibodies (IgG), proving that folates may only be active if they are transported when linked to molecules that promote stabilization. The present study demonstrates the suitability of stabilized THF as a potential candidate for treatment of hydrocephalus.

- FDH PROTEIN IS UNDEREXPRESSED IN HYDROCEPHALIC CEREBRAL CORTEX.

Protein expression at tissue levels is decreased in the hydrocephalic cerebral cortex, whereas FRα and 5mTHF are increased, implying that there is a compensatory mechanism to provide the cerebral cortex with 5mTHF through an elevated production of FRα as FDH is low. However, since FRα is not synthesized in the brain, we suggest that the FRα-5mTHF folate delivery system is controlled by the placenta, indicating that this auxiliary fetal organ is impaired in hydrocephalus. Our FDH protein expression in brain tissue agrees with past results from CSF analysis in our lab where FDH appears to be decreased in hydrocephalic CSF.

- FDH PROTEIN EXPRESSION DOES NOT CORRELATE WITH mRNA EXPRESSION LEVELS IN THE HYDROCEPHALIC BRAIN.

FDH mRNA/cDNA expression quantification did not correlate with the high levels of protein found, which indicates that mRNA transcription and/or translation processes may be altered in congenital hydrocephalus, resulting in low FDH protein biosynthesis at tissue level and later secretion into CSF. On the other hand, FDH high mRNA expression in hydrocephalic cerebral cortex suggests a negative feedback loop to compensate for a decreased FDH protein biosynthesis.

- PROSPECTIVE ROLE OF FDH IN CELL METHYLATION STATUS

The ability of FDH to bind to 5mTHF also implies a role in the provision of methyl groups for diverse metabolic reactions, such as DNA methylation, production of the universal methyl group donor SAM, as well as the production of methionine. Because balanced levels of methionine and homocysteine are crucial for development, a low methyl group bioavailability due to low 5mTHF provision by FDH means homocysteine is in excess. High levels of homocysteine is associated with hypomethylation and cell cycle arrest, as well as damage to the interior lining of the blood vessels walls like the carotid arteries in the brain. Furthermore, increased homocysteine can have a negative impact in placenta formation, and may lead to abruption as well as pre-eclampsia during pregnancy.

### 5.1.3. <u>Future aims</u>

☐ Further FDH protein and gene expression quantification in relation to FRα and 5mTHF in cerebral cortex, choroid plexus and placenta could help to confirm the extent to which FDH and FRα gene expression is upregulated in congenital hydrocephalus. Whereas in this study RTPCR allowed gene expression quantification in normal and abnormal cerebral cortex, this technique was not used with choroid plexus tissue due to technical problems related to the concentration of this tissue. Since fluorescence "in situ" RTPCR allows location as well as quantification of mRNA expression in both choroid plexus and cerebral cortex, this technique is proposed as more covenient for our purposes to quantify FDH and FRα mRNA in normal and abnormal brain. In relation to localization, fluorescence immunostaining allowed location and quantification of FDH and FRα proteins; however, further studies using Western Blotting are also suggested for double confirmation of this result.

☐ Past studies revealed a combination of two folates (THF and Folinic acid) as a poential suitable treatment for congenital hydrocephalus. Whilst Folinic acid on its own does not reduce the incidence of this condition in Texas rats animal models, THF did encourage cell growth "in vitro", highlighting the importance of this folate as a suitable candidate for treatment. Further "in vivo" experiments using stabilized THF, either with specific anti-THF IgG or FDH to Texas rats, should be carried out to ascertain whether there is any reduction in the incidence of congenital hydrocephalus with this folate.

☐ High homocysteine levels are thought to be ocurring in hydrocephalic CSF due to low bioavailability of methyl groups caused by low FDH and therefore 5mTHF provision, leading to low methionine production. High homocysteine and low methionine levels in plasma are linked to high blood pressure, hypomethylation and placental and brain developmental problems. Thus, a future aim in this research should be the measurement of homocysteine concentration in hydrocephalic CSF to determine whether this is raised compared to normal CSF.

**5.2.1. Chapter 3 concluding remarks flow chart. Figure 5.2:**



LCMS ANALYSIS OF NORMAL AND HC CSF

+ FDH IN NORMAL CSF

− FDH IN HC CSF

− FDH IN HC CSF SUGGESTS IMBALANCED FOLATE METABOLISM

FDH/FOLATES EFFECT ON ARACHNOID CELL PROLIFERATION: "IN VITRO" STUDIES

NORMAL AND HC CSF EFFECT ON ARACHNOID CELL PROLIFERATION: "IN VITRO" STUDIES

ONLY 5mTHF PROMOTES CELL GROWTH AND THIS IS DIRECTLY PROPORTIONAL TO THE FOLATE CONCENTRATION IN THE MEDIA

CSF ENCOURAGES CELL PROLIFERATION AND HC CSF PRESENTS THE GREATEST EFFECT ON CELL DIVISION AND DIFFERENTIATION

THESE RESULTS SUGGEST CONGENITAL HYDROCEPHALUS MAY BE DUE TO DEVELOPMENT OF A DYSFUNCTIONAL ARACHNOID CAUSED BY ↓ FDH IN HC CSF (LCMS ANALYSIS) LEADING TO 5mTHF LEVELS' DEREGULATION LEVELS BY FDH AND ITS ACCUMULATION IN CSF (↑5 mTHF WAS OBSERVED IN PAST WESTERN BLOTT STUDIES).

↑5 mTHF in CSF MAY ULTIMATELY LEAD TO UNCONTROLLED CELL DIVISION RESULTING IN AN ABNORMAL ARACHNOID TISSUE UNABLE TO DRAIN CSF INTO THE BRAIN BLOOD VENOUS SINUS.

HC: HYDROCEPHALIC

## 5.2.2. Chapter 3 concluding remarks and discussion

▢ FOLATES' EFFECT ON CELL DIVISION ON ARACHNOID PRIMARY CELL CULTURES REVEALED 5MTHF AS THE ONLY FOLATE ABLE TO ENCOURAGE CELL ARACHNOID PROLIFERATION AND DIFFERENTIATION.

5mTHF potentiated cell to cell connections, which suggests that this folate is needed for flow regulation through cell juntions and is vital for the correct development of a functional aracnoid in early pregnancy.

▢ 5MTHF AT HIGH CONCENTATION WAS COUNTERPRODUCTIVE FOR CELL GROWTH IN THE PRESENCE OF CONSTANT 5mTHF AND ACTIVE FDH AND VICE VERSA.

These outcomes provide support for previous "in vitro" work about the effects of folates on cerebral cortex cells. In conclusion, these results reaffirm FDH's affinity for 5mTHF, pooling this folate when in excess, and acting as a regulatory enzyme to store it in situations of low demand. Therefore, a lack of FDH leads to increased 5mTHF levels in hydrocephalic CSF.

▢ PRIMARY ARACHNOID CELL CULTURE IS HIGHLY PROMOTED BY HYDROCEPHALIC CSF.

This outcome corroborates the impact of high levels of 5mTHF on arachnoid cell proliferation in the absence of FDH (HC CSF composition), providing further evidence about the role of FDH in 5mTHF regulation levels in CSF.

### 5.2.3. <u>Chapter 3 future aims</u>

☐ In the present study, all "in vitro" work was performed on arachnoid tissue from normal brain. Future experiments to study the effect of folates as well as normal CSF on arachnoid primary cell cultures from hydrocephalic fetal brain may confirm further whether 5mTHF is beneficial for primary hydrocephalic arachnoid cell culture.

☐ Further FDH and FRα protein and gene expression analysis on arachnoid tissue may also contribute to a better understanding of the role of folate transport and folate metabolism in the development of the fetal arachnoid during early pregnancy in normal and abnormal brain.

**5.3.1 Chapter 4 concluding remarks flow chart. Figure 5.3:**



| LCMS ANALYSIS OF NORMAL AND HC CSF |
|---|

| + FDH IN NORMAL CSF | | - FDH IN HC CSF |
|---|---|---|

| EXCEL ANALYSIS TO GROUP PROTEINS UNIQUELY FOUND IN NORMAL CSF | EXCEL ANALYSIS TO GROUP PROTEINS UNIQUELY FOUND IN HC CSF |
|---|---|

| IPA ANALYSIS TO IDENTIFY PROTEINS UNIQUE TO NORMAL CSF AND ASSOCIATED TO SPECIFIC METABOLIC PATHWAYS | IPA ANALYSIS TO IDENTIFY PROTEINS UNIQUE TO ABNORMAL CSF AND ASSOCIATED TO SPECIFIC METABOLIC PATHWAYS |
|---|---|

| CANONICAL PATHWAY | MOLECULES |
|---|---|
| Glutamine degradation I | GLS |
| Uracil degradation II | DPYD |
| Thymine degradation | DPYD |
| Geranylgeranyl diphosphate biosynthesis | FDPS |

| CANONICAL PATHWAY | MOLECULES |
|---|---|
| Folate polyglutamation | MTHFD1 SHMT1 |
| Superpathway of serine & glycine biosynthesis I | SHMT1 PSAT1 |
| Calcium transport I | ATP2A2 ATP2C1 ATP2C2 |
| Folate transformations I | MTHFD1 SHMT1 |

IPA ANALYSIS GENERATED POTENTIAL METABOLIC PATHWAYS THAT MAY BE ACTIVATED/INACTIVATED IN NORMAL AND HC CSF RESPECTIVELY. HOWEVER, GENETIC EXPRESSION ANALYSIS OF THE MOLECULES INVOLVED IN THESE PATHWAYS IS NEEDED TO REVEAL ANY GENE'S DIRECTIONALTY

### 5.3.2. Chapter 4 concluding remarks and discussion

☐ IPA ANALYSIS OF NORMAL CSF ALLOWED THE DETECTION OF THE ENZYME FDH WHICH FALLS WITHIN THE FOLATE TRANSFORMATION I PATHWAY AND WHICH WAS ABSENT IN ABNORMAL CSF.

FDH is part of the Folate transformations I pathway where FDH transforms 10-formylTHF into THF. This outcome is in agreement with past Western blotting results that showed a lack of FDH in hydrocephalic CSF suggesting this pathway may be missing in the folate metabolic cycle leading to accumulation of 10-formylTHF and a lack of THF synthesis, which is crutial for the production of DNA. On the other hand, it is thought that FDH may also have a role in regulation of 5mTHF levels through its binding to 5mTHF for storage in situations of high 5mTHF concentration in CSF. Thus, depletion of FDH may also lead to increased levels of free 5mTHF in CSF and abnormalities in cell divison rate. This conclusion is supported first by past Western Blotting studies that revealed abnormal high levels of 5mTHF in hydrocephalic CSF and second, by our "in vitro"studies that showed how cell division is directly proportional to the 5mTHF concentration in the media.

☐ IPA ANALYSIS OF NORMAL CSF ALLOWED THE DETECTION OF THE ENZYME FDH WHICH FALLS WITHIN THE FOLATE TRANSFORMATION I PATHWAY AND WHICH WAS ABSENT IN ABNORMAL CSF. GLS IN THE GLUTAMINE DEGRADATION PATHWAY

GLS is involved in the glutamine degradation pathway and was exclusively found in normal CSF. IPA associations between molecules uniquely found in normal CSF and the glutamine degradation pathway were significant at $p < 0.05$. According to

this outcome, we postulate that glutamine degradation is activated in normal CSF, but inactivated in abnormal CSF, implying that this metabolic pathway and associated biological processes are impaired in hydrocephalus, with negative consequences for brain development as a lack of glutamine degradation means no prodcution of the key neurotransmitor glutamate.

☐ IPA ANALYSIS CREATED ASSOCIATIONS BETWEEN MOLECULES ONLY PRESENT IN HYDROCEPHALIC CSF AND FOLATE POLYGLUTAMATION (MTHFD1 AND SHMT1), THE SUPERPATHWAY OF SERINE AND GLYCINE BIOSYNTHESIS I (PSAT1 AND SHMT1) AND CALCIUM TRANSPORT I (ATP2C2, ATP2C1, ATP2A2) AS WELL AS FOLATE TRANSFORMATIONS I PATHWAYS (MTHFD1 AND SHMT1)

In relation to these results we hypothesize that these metabolic pathways are activated exclusively during congenital hydrocephalus at gestational age 18. Interestingly, SHMT1 was an enzyme present in most of the metabolic pathways associated with hydrocephalic CSF, which highlights this protein as a key molecule involved in the onset of congenital hydrocephalus.

### 5.3.3. Chapter 4 future aims

Drawbacks like undersampling are known features of LCMS technology, which means that there was possible underdetection of small molecules or molecules present at low concentration in the CSF samples used in our experiments. Therefore, we propose further experiments involving CSF fractionation and new mass spectrophotometry analysis using cutting-edge technologies like 6600 Triple ToF mass spectrophotometry, followed by IPA re-analysis in parallel to quantification of gene expression of all molecules associated with

metabolic pathways identified by IPA analysis. This approach will result in a more detailed and exhaustive study of normal and abnormal to establish CSF differences and to determine which genes are activated and inactivated in congenital hydrocephalus.