Physiopathology of Foetal Onset Hydrocephalus

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Abbreviations

AQP4	Aquaporin 4
CSF	Cerebrospinal fluid
GW	Gestational week
NPC	Neural progenitor cell
NSC	Neural stem cells
SA	Sylvius aqueduct
SVZ	Subventricular zone
VZ	Ventricular zone

Balanced View of CSF Physiology

Aiming to a Balanced View of CSF Physiology

Several functions have been ascribed to the cerebrospinal fluid (CSF), including protection to the brain, excretion of metabolites, homeostasis of the brain chemical environment, and as a transport pathway between different brain areas [18, 80, 98, 108]. These various functions, coupled with its rapid turnover, perpetual formation,

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and continuous circulation and absorption have led to consider the CSF as the "third circulation," as first referred to by Cushing [16].

For decades, the CSF was regarded as a water solution of ions and few other components, such as glucose and vitamins. The concept of waste drainage was also associated to the "physiology" of CSF. Furthermore, the functional significance of the complex structure of the ventricular and subarachnoid compartments and the multiple populations of cell types lining discrete areas of the ventricular walls were, and still are, overlooked or neglected.

When the cerebrospinal fluid-contacting neurons were discovered, the concept that the CSF could be a pathway for signal molecules started to develop. This idea was strongly substantiated by the demonstration that the choroid plexus is a true gland that, in addition to transport water and ions, it also has the capacity to transport peptides and proteins from blood to CSF and to synthesize and secrete into the CSF a series of biologically active molecules [14, 78, 98]. Although the series of peptides, proteins, and neurotransmitters detected in the CSF using different methods increased throughout three decades, it was the analysis by mass spectrometry that suddenly revealed the enormous complexity of the molecular composition of the CSF.

In recent years, the discovery of aquaporins and other water transporters, all highly selective for water molecules, has again moved the balance to the oversimplified view that CSF physiology refers almost exclusively to water exchange between brain compartments. The glymphatic concept emphasizes the transport of water and waste molecules from the brain parenchyma into subarachnoid space along perivascular pathways of the Virchow Robin spaces, overlooking the fact that this "brain parenchyma" refers to the most superficial region of the brain cortex. It is really disturbing when the physiology of CSF is only associated with the movement of water through the different brain compartment, what leads several authors to talk about CSF secretion when in actuality they are only referring to water transport, completely disregarding the rich heterogeneity of the ventricular walls (circumventricular organs included) and the wealth of signal molecules that use the CSF as a pathway.

The CSF as a Pathway for a Cross Talk Between Different Periventricular Regions

CSF proteomics is showing a wealth of over 200 proteins [113]. A long series of peptides and neurotransmitters are also present in the CSF. Some of these compounds move by bulk flow from the interstitial fluid of brain parenchyma, many are secreted by neurons, glia, and ependyma into the CSF, others are transported by specific transport systems from blood to ventricular CSF (choroid plexus) while a few of them originate from cells present in the CSF. For many of these compounds, CSF levels bear hardly any relationship to peripheral levels in the blood [98].

The long series of biologically active proteins, peptides, and neurotransmitters present in the CSF reach this fluid through different mechanisms: (1)

Neurotransmitters and their metabolites reach the CSF via the bulk flow of parenchymal fluid. (2) Regulated secretion into the CSF of biologically active compounds by the circumventricular organs (subcommissural organ, pineal gland, choroid plexuses, and median eminence), such as SCO-spondin, basic fibroblast growth factor, melatonin, transthyretin, transthyretin-T4 complex, transthyretin-T3 complex, nerve growth factor (NGF), transforming growth factor-β (TGFβ), vascular endothelial growth factor (VEGF), transferrin and vasopressin [28, 42, 43, 81]. (3) Selective and circadian regulated secretion by CSF-contacting neurons of serotonin and neuropeptides such vasopressin, oxytocin, and somatostatin [80, 99, 100]. (4) Transport of peripheral hormones through the choroid plexus. Most of the transported hormones, such as leptin, prolactin, and thyroxin, have specific targets, mostly the hypothalamus [14, 81]. The concentrations of these neuroactive compounds vary between locations, suggesting they are important for the changes in brain activity that underlie different brain states [98].

Furthermore, a series of findings indicate that cells forming the ventricular walls release into the CSF microvesicles containing signalling and intracellular proteins [13, 22, 32, 55, 96]. Harrington et al. [32] suggested that this bulk flow of nanostructures generates a dispersed signal delivery, of longer duration.

Thus, the early view that the CSF is a medium carrying brain-borne and blood-borne signals to distant targets within the brain [80] has largely been supported by numerous investigations [42, 45, 81, 108]. Worth mentioning here is the much neglected system of CSF-contacting neurons most likely playing receptive functions sensing CSF composition. Most of these neurons are bipolar with the dendritic process reaching the CSF and endowed with a 9 + 0 single cilium [100].

In brief, a good body of evidence is revealing that the dynamic and molecular composition of the CSF and, consequently, the CSF physiology is much more complex and fascinating than the simplistic view held for decades. Signal molecules, either specifically transported from blood to CSF or secreted into the CSF by a series of periventricular structures, use the CSF to reach their targets in the brain. This allows a cross talk between brain regions located beyond the blood-brain-barrier, thus keeping the brain milieu private [29, 98].

Changing of CSF Composition as It Moves Through the Ventricular System

The ventricular CSF changes its molecular composition as it unidirectionally moves through the various ventricular and subarachnoid compartments (Fig. 1.1a). The choroid plexus of the lateral ventricles, the interstitial fluid of the parenchyma surrounding these ventricles, and axon endings secreting into these cavities are the source of molecules forming this “first” fluid. At the third ventricle, new compounds are added to the CSF by hypothalamic neurons, the pineal gland, and the local choroid plexus [42, 69, 80]. When entering the Sylvius aqueduct (SA), the CSF is enriched by the secretion of the subcommissural organ [101] (Fig. 1.1a). Consequently, the CSF of the fourth ventricle is different as compared to that of the

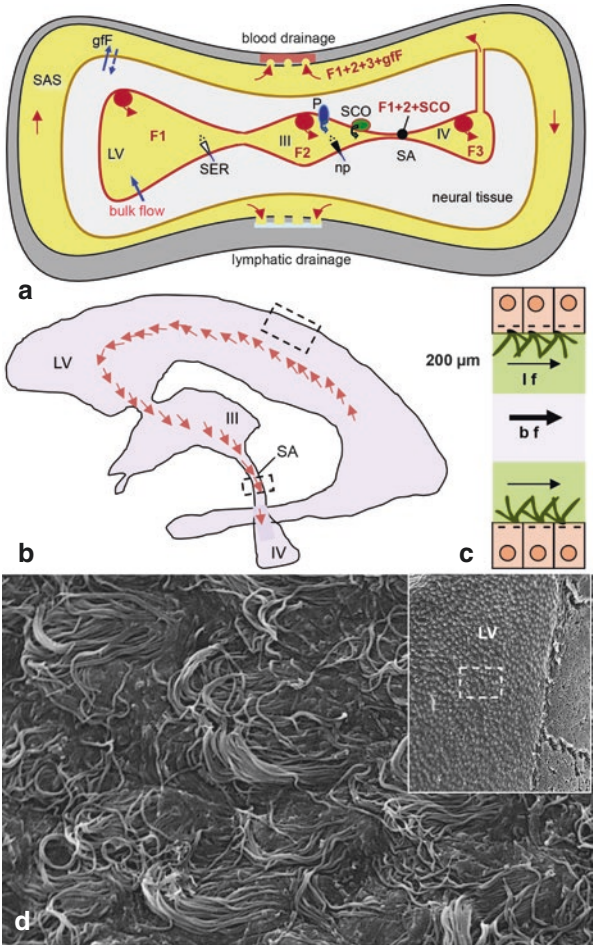


Fig. 1.1 (**a**) Line drawing depicting the changes in the CSF compositions as it moves throughout the lateral (LV), third (III) and fourth (IV) ventricles. F1 = CSF of the lateral ventricles contains molecules from the bulk flow of the parenchyma and compounds secreted by the choroid plexus and CSF-contacting neurons, such as serotonin (SER). F2 = CSF of the third ventricle contains F1 plus compounds secreted by the choroid plexus and hypothalamic CSF-contacting neurons (np, neuropeptides) and the pineal (P). SA = the Sylvius aqueduct contains F1 + F2 plus the secretion of the subcommissural organ (SCO). F3 = CSF of the fourth ventricle contains F1 + F2 + SA fluid plus compounds secreted by the choroid plexus and CSF-contacting neurons. The CSF of the subarachnoid space contains F1 + F2 + F3 plus molecules from the glymphatic flow (gfF) draining the most superficial region of the brain cortex. (**b**) Line drawing representing the laminar CSF flow (arrows) generated by the cilia beating. (**c**) Scheme representing the laminar (lf) and bulk (bf) of CSF though the Sylvius aqueduct (for orientation see rectangle in **b**). The broken line on top of ependymal cells depicts the negative charges from sialoglycoproteins of the glycocalix. (**d**) Scanning electron microscopy of the lateral wall of a normal rat showing the bundles of cilia of each cells beating in the same direction. *Inset.* Low SEM magnification of a lateral wall of a lateral ventricle (LV). Rectangle frames an area similar to that shown in **d**

lateral ventricles [113]. This partially explains the different protein composition between the CSF collected from the lateral ventricles and that obtained from a subarachnoid compartment [101].

Furthermore, at the interphase brain cortex/subarachnoid space there is a bidirectional flow of CSF and interstitial fluid along the large paravascular spaces that surround the penetrating arteries and the draining veins (Fig. 1.1a). Since water movement along this pathway is mediated by astroglial aquaporin-4 (AQP4) water channels, this paravascular pathway has been termed "glymphatic system" [35, 36]. This pathway facilitates efficient clearance of interstitial solutes, and its failure may lead to neurodegeneration [37].

Multiciliated Ependyma and CSF Flow

The mechanisms responsible for the CSF circulation are not fully understood. The following factors do play a role: (1) the hydrostatic difference between the production and drainage sites; (2) the pulsations of the cerebral arterial tree; (3) the directional beating of ependymal cilia [18, 106, 107]. The relative contribution of each of these forces is still controversial.

The flow of the CSF throughout the ventricular system involves two different mechanisms, the bulk flow and the laminar flow. Bulk flow is driven by arteriovenous pressure gradients and arterial pulsations. The laminar flow occurs in a thin layer along the walls in a variety of directions [98]. It has been shown that cilia beating is responsible for the laminar flow of CSF, whereas its role in the bulk of CSF taking place in the core of the ventricular cavities is probably insignificant [15, 59, 66, 106]. The cilia beating of the ependyma of the lateral ventricles generate currents as far as 200 μm away from the surface [66] (Fig. 1.1b–d). In the frog brain, about 75% of the CSF within the ventricles is mixed as a result of ciliary activity [66]. Ciliary currents adjacent to the ependyma have been observed in rats, dogs, and humans [109].

The ciliary beating is supported by a sialic acid-induced hydration mantle on the ependymal surface [98]. The frequency of cilia beating is stimulated by the activation of serotonin receptors [68]. Serotonin is released by axon terminals of the suprapendymal serotonergic plexus originated in raphe nuclei [12, 68, 102].

Using computational fluid dynamics, the relative impact of macroscale (choroid plexus pulsation and ventricular wall motion) and microscale (beating of cilia) effects on near-wall CSF dynamics has been investigated [95]. This study revealed a marked effect of the cilia on the near-wall dynamics and directionality but not on the bulk flow. Conversely, the bulk flow alone does not produce any notable directionality of the flow near or on the surface of the lateral ventricles. The authors concluded that in the lateral ventricles, near-wall CSF dynamics is dominated by ependymal cilia action [95]. This confirms early observations that the ciliary movement plays a key role in the maintenance of an adequate CSF flow [31, 109, 111]. The role of the multiciliated ependyma in CSF dynamics is strongly supported by the demonstration that primary cilia dyskinesia, a syndrome that impairs ciliary

activity, leads to the development of hydrocephalus [2, 17, 27, 34, 50, 51, 97]. Shimizu and Koto [93] have suggested that immotility of cilia is of particular importance in narrow canals, such as the Sylvius aqueduct, for the development of hydrocephalus.

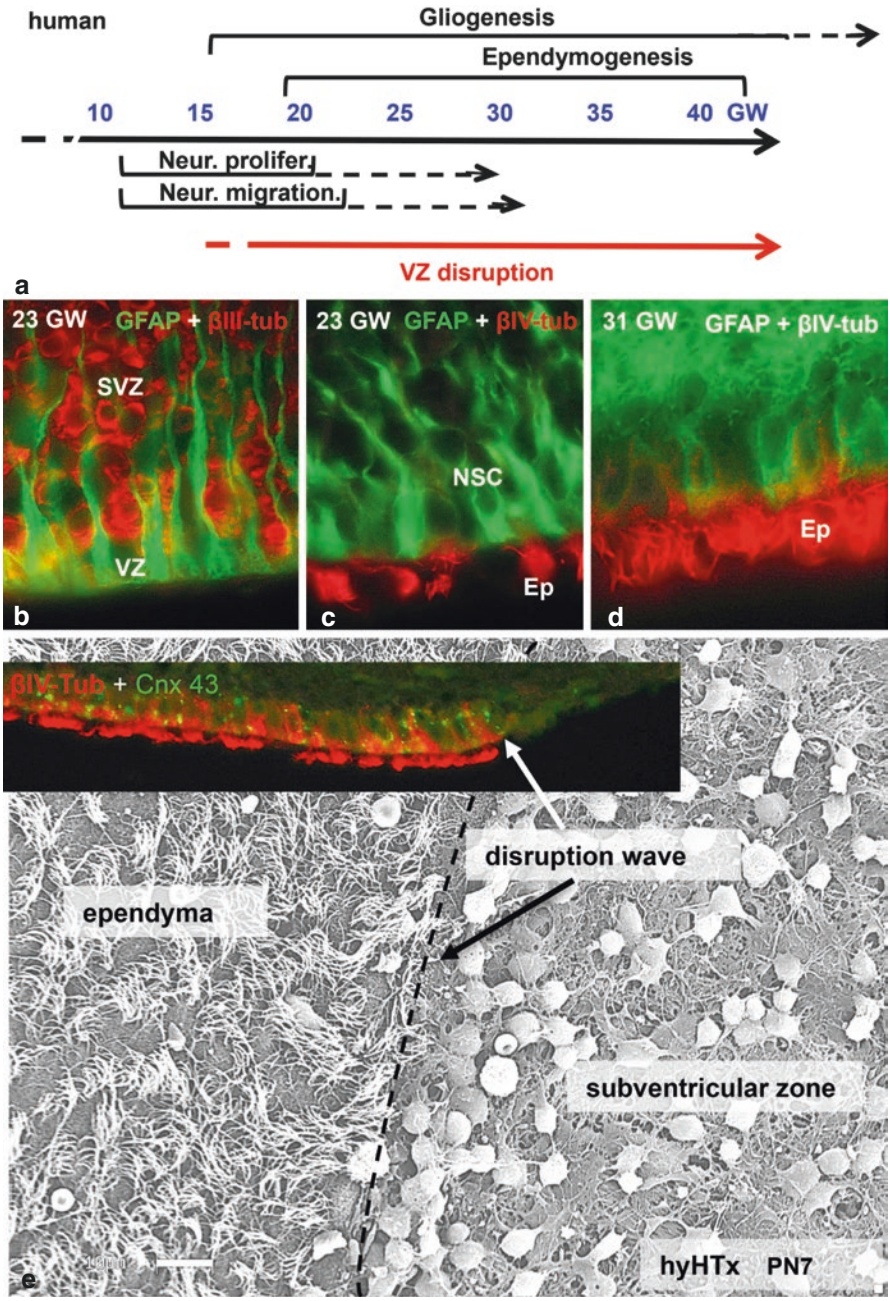
Human Ependymogenesis

The Wall of the Lateral Ventricles of the Human Developing Brain Is a Complex and Dynamic Mosaic

Very little, if any, attention has been paid to the complexity of the cell organization of different domains of the ventricular walls. To make it even more complex, such a mosaic undergoes changes during foetal development. This dynamic complexity of the ventricular walls partially resembles that of the ventricular walls of the rodent developing brain. During the last few day of foetal life, the medial wall of the lateral ventricles of the rat is already lined by multiciliated ependyma while the lateral and dorsal walls continue formed by neural stem cells (NSC) [29]. In human embryos, there is also an early division of labour between the medial and the latero-dorsal walls of the lateral ventricles [57]. Such a partition of labour seems an efficient design; while the latter is permanently involved in neurogenesis, the medial wall is progressively engaged in the flow of CSF. Indeed, coupled multiciliated ependymal cells generate the laminar flow of CSF [59, 66] that is essential for the CSF flow along the ventricular system (see above).

In the human developing telencephalon, ependymogenesis starts about the 18th gestational week (GW) (Fig. 1.2a) in the medial wall of the lateral ventricles and progressively continues through the lateral and then to the dorsal walls [57, 83]. Thus, in young foetuses, the lateral wall of the ventricle is fully involved in neurogenesis while the medial wall starts to change its role, from neurogenesis to ependymogenesis. In pre-term foetuses, while the medial wall is fully lined by multiciliated mature ependyma, the lateral wall has mixed populations of cells suggestive of neurogenesis and ependymogenesis.

Fig. 1.2 (**a**) Panel summarizing the timetable of key events in the developing human brain. The bulk of neural proliferation and neural migration occurs between 12 and 22 GW; then both processes decrease progressively. The process of ependymogenesis starts at about 18th GW and is completed after birth. Gliogenesis starts at about the 15th GW and continues for several months after birth. In the hydrocephalic, human brain disruption of the ventricular zone starts at about 17 GW (*red arrow*). (**b**, **c**) Photomicrographs of a hydrocephalic foetus (23 GW) illustrating the VZ formed by two types of cells: radial GFAP-positive cells (NSC) and radial GFAP/βIV-tubulin positive cells. The subventricular zone (SVZ) contains βIII-tubulin+ progenitor cells. (**d**) The VZ of a 31 GW hydrocephalic foetus appears formed by multiciliated βIV-tubulin+ ependyma (Ep). (**e**) Hydrocephalic HTx rat, PN7. Scanning electron microscopy of the dorsal wall of a lateral ventricle showing the disruption wave, leaving the subventricular zone nude. *Inset.* Similar region with double immunofluorescence for βIV-tubulin (multiciliated ependyma) and connexin 43 (gap junctions). (Source: **a**–**d** from [83])


human
Gliogenesis
Ependymogenesis
10
15
20
25
30
35
40 GW
Neur. prolifer.
Neur. migration.
VZ disruption
a
23 GW GFAP + βIII-tub
SVZ
VZ
b
23 GW GFAP + βIV-tub
NSC
Ep
c
31 GW GFAP + βIV-tub
Ep
d
βIV-Tub + Cnx 43
disruption wave
ependyma
subventricular zone
hyHTx PN7
e

Cell Types of the VZ. Evidence for an Ependymogenesis Program

According to Rakic [76], the human telencephalic proliferative zone contains considerably complex progenitor cell groups that change during the course of development. In young embryos (5–6 GW) the VZ contains a mixed population of cells; most of them express neural stem markers only (GFAP, GLAST) while others, in addition, also express neuronal markers (βIII-tubulin, MAP-2) indicating their multipotential capacity [114]. Later in development (10–22 GW), vimentin +, GFAP+ cells displaying a long basal process persisted. Proliferation of GFAP+ cells of the ventricular zone (VZ) occurs until the 23rd GW, coinciding with the formation of the ependyma [114]. According to Gould et al. [26], in early pregnancy, the VZ is formed by GFAP+ radial glia/neural stem cells, whereas in late pregnancy, the VZ is formed by GFAP+ ependymal cells with a short basal process,

By use of several markers, Sarnat [85, 86, 88] has followed in human foetuses the temporal and spatial differentiation of cells lining the ventricular walls. In these studies, Sarnat has regarded as ependyma all cells lining the foetal ventricular system. Using this criterion, he concluded that the expression of GFAP and vimentin in foetal ependymal cells follows a regional and temporal distribution [85, 86], with the ependyma of the roof and floor plates being the first to differentiate. During several gestational weeks, GFAP is co-expressed with vimentin in most foetal ependymal cells. At birth, only scattered ependymal cells of the lateral ventricles still express GFAP, and it disappears entirely within the first few weeks of postnatal life [88].

The true process of ependymogenesis in the human remains largely unknown due, to a great extent, to limitations to obtain samples of the ventricular walls from systematically selected regions and selected gestational ages, and to process these samples for different methods. A recent investigation has provided some new evidence on ependymogenesis [57]. Based on the immunoreactivity to GFAP, AQP4, βIV-tubulin, and βIII-tubulin, their morphology (basal process, one or multiple cilia) and their spatial and temporal distribution, we have distinguished seven cell types in the VZ of the lateral ventricles of human foetuses [57, 83]. Type 1 cells with a long radial process, expressing AQP4 in the plasma membrane domain and GFAP throughout the cytoplasm, displaying a single cilium, is the main cell type in the VZ of young foetuses and most likely correspond to NSC (Fig. 1.2b). Type 2 cells are identical to type 1 cells but also express βIV-tubulin, a well-known marker of multiciliated ependyma, suggesting they correspond to NSC that have started to differentiate into ependymal cells (Fig. 1.2c). Cells type 3 through 6 would reflect progressive stages of ependymal differentiation ending in the differentiated multiciliated, βIV-tubulin+, ependyma (type 7) present during the last trimester of foetal life and throughout adulthood (Fig. 1.2d).

Hydrocephalus

A Concept

Foetal-onset hydrocephalus is a heterogeneous disease. Genetic and environmental factors, such as vitamin B or folic acid deficiency [40], viral infection of ependyma [44], and prematurity-related germinal matrix and intraventricular haemorrhage [7], contribute to its occurrence.

Numerous investigations in humans and mutant animals have substantiated the view that hydrocephalus is not only a disorder of CSF dynamics but also a brain disorder, and that derivative surgery does not resolve most aspects of the disease [46]. Actually, 80–90% of the neurological impairment of neonates with foetal onset hydrocephalus is not reversed by derivative surgery. *How can we explain the inborn and, so far, irreparable neurological impairment of children born with hydrocephalus?* In 2001, Miyan and his co-workers asked a key question [60]: "Humanity lost: the cost of cortical maldevelopment in hydrocephalus. Is there light ahead?" Although this appealing question has not been responded, there is some light in the horizon. A strong body of evidence indicates that the common past of hydrocephalus and brain maldevelopment starts early in the embryonic life with the disruption of the ventricular (VZ) and subventricular (SVZ) zone. However, the nature, mechanisms, and extent of the brain impairment linked to hydrocephalus are far from been fully unfolded. Certainly, a better treatment of hydrocephalus and the associated neurological impairment will come from a better understanding of the biological basis of the brain abnormalities in hydrocephalus [19, 105]. This view may represent one of the "lost highways" in hydrocephalus research, as described by Jones and Klinge [46].

To have clarity of the timetable of neurogenesis and ependymogenesis in normal rodents and humans seems essential for a better understanding of the early events occurring in foetal onset hydrocephalus.

Prenatal Neurogenesis. Timetable of Neural Proliferation and Migration, Gliogenesis, and Ependymogenesis

Virtually, all cells of the developing mammalian brain are produced in two germinal zones that form the ventricular walls, the VZ and the SVZ [6, 8, 25, 58, 39, 53]. The VZ is a pseudostratified neuroepithelium that contains multipotent radial glia/stem cells, hereafter called neural stem cells (NSC). NSC line the ventricular lumen and through a long basal process reach the pial surface. A landmark of NSC is their primary cilia that project to the ventricle and are bathed by the foetal CSF [47, 64]. During a fixed period of brain development, NSC divide asymmetrically, with one daughter cell remaining as a NSC and the other becoming a neural progenitor cell (NPC). NPC proliferate and cluster underneath the VZ, forming the so-called

SVZ. NPC differentiate into neuroblasts that start migration using the basal process of NSC as scaffold. In the human, the bulk of neural proliferation and neuroblast migration occurs at a rather short period, between GW 12 and 18 (Fig. 1.2a).

Gliogenesis starts at about the 15th GW and continues for several months after birth. Ependymal cell differentiation starts at about the 18th GW and is completed after birth (Fig. 1.2a) [83, 85, 86].

Over the years, based on our own and other investigators' evidence, we have progressively come to the view that a *disruption of the VZ and SVZ*, in most cases due to genetic defects, triggers onset of congenital hydrocephalus *and* abnormal neurogenesis (Fig. 1.2a, c). We will discuss this evidence below.

Brain Damage Versus Brain Defects

A distinction must be made between (1) brain *maldevelopment* due to a primary pathology of the VZ that precedes or accompanies onset of hydrocephalus and (2) brain *damage* caused by hydrocephalus. The former occurs during development, and consequently neonates *are born* with a neurological deficit. Brain *damage* is mainly a postnatal acquired defect, essentially caused by ventricular hypertension and abnormal CSF flow and composition.

Brain damage may be associated to regional ischemia, disruption of white matter pathways, and alteration of microenvironment of neural cells [19, 20]. Derivative surgery, the almost exclusive treatment of hydrocephalus today, is aimed to prevent or diminish brain *damage*. It is clear that hydrocephalic patients improve clinically after surgery due to correction of intracranial pressure, improvement in white matter blood flow [19], and probably to resumption of the clearance role of CSF. However, *derivative surgery does not reverse the inborn brain defects*. This has led a study group on hydrocephalus to conclude that "Fifty years after the introduction of shunts for the treatment of hydrocephalus, we must acknowledge that the shunt is not a cure for hydrocephalus" [5].

Ventricular Zone Disruption

A Concept and Definitions

For clarity purposes, we shall define the terms used in the present chapter to refer to the ventricular zone. At stages of development when the VZ is mostly formed by neural stem cells (NSC), the acronym VZ will be used. When the VZ is mostly or exclusively formed by multiciliated ependymal cells, the term "ependyma" will be used. The terms "denudation," "disruption," or "loss" will be alternatively used to refer to the disassembling, disorganization, or loss of the VZ cells [81]. A solid body of evidence indicates that radial glial cells are neural stem cells. Throughout the present text, we shall use the term neural stem cells, and its acronym NSC, to refer to the cells forming the embryonic ventricular zone, characterized by a long basal

process, a single 9 + 0 cilium projecting to the ventricle and by expressing certain markers such a nestin [57].

In mutant animals, the disruption of the VZ follows a program that has temporal and spatial patterns, progressing as a "tsunami" wave running from caudal to rostral regions of the developing ventricular system, leaving behind a severe damage (Fig. 1.2e) [29, 41, 74, 81, 103]. A similar process of VZ disruption occurs in human hydrocephalic foetuses [21, 29, 82, 83, 94]. Since the VZ disruption is a continuous process, starting during the embryonic life and continuing during the first postnatal week, the pathology first affects NSC, then the NSC differentiating into ependymal cells and finally the differentiated multiciliated ependyma. These three cell types have distinct phenotypes and certainly play quite different roles. What do they have in common so that the denudation wave will hit them all? Junction proteins appear to be the key to understanding this devastating phenomenon [29].

A Stormy Intracellular Traffic of Junction Proteins in NSC and Ependymal Cells Leads to Ventricular Zone Disruption

What is the molecular mechanism underlying the VZ disruption occurring in human hydrocephalic foetuses, the HTx rat and in various mutant mice developing hydrocephalus? Overall, a series of findings indicates that disruption of VZ arises from a final common pathway involving alterations of vesicle trafficking, abnormal cell junctions, and loss of VZ integrity [23, 38, 48, 52, 77]. The abnormal localization of N-cadherin and connexin 43 in NSC and ependymal cells and the formation of subependymal rosettes suggest that VZ disruption results from a defect in cell polarity and in cell–cell adhesion of VZ cells. The accumulation of N-cadherin and connexin 43 in the soon-to-detach VZ cells and their virtual absence from the plasma membrane indicate that they are synthesized by the disrupting cells but are not properly transported to the plasma membrane (Fig. 1.3a–d) [29]. The mechanism actually involved in this abnormal expression and translocation of N-cadherin is unknown. The specific disruption of N-cadherin-based junctions is enough to induce ependymal disruption. Indeed, antibodies against chicken N-cadherin injected into the CSF of chick embryos disrupt the VZ, lead to denudation of the SVZ and formation of periventricular rosettes [24]. Similarly, the use of N-cadherin antibodies or synthetic peptides harbouring a cadherin-recognition sequence triggers the detachment of ependymal cells from explants of the dorsal wall of the bovine Sylvius aqueduct [71]. The abnormal localization of connexin 43 in the NSC and ependymal cells could be associated to the faulty localization of N-cadherin. Indeed, it has been reported that gap junction proteins are delivered to the plasma membrane at adherent junction sites [90].

In mutant mice, several gene mutations leading to abnormal trafficking of junction proteins and resulting in VZ disruption have been reported [11, 38, 41, 48, 52, 54, 91]. The nature of the genetic defect in hydrocephalic patients [21, 72, 94] is unknown. It may be postulated that they all carry a defect at one or another point of the pathways assembling adherent and gap junctions.

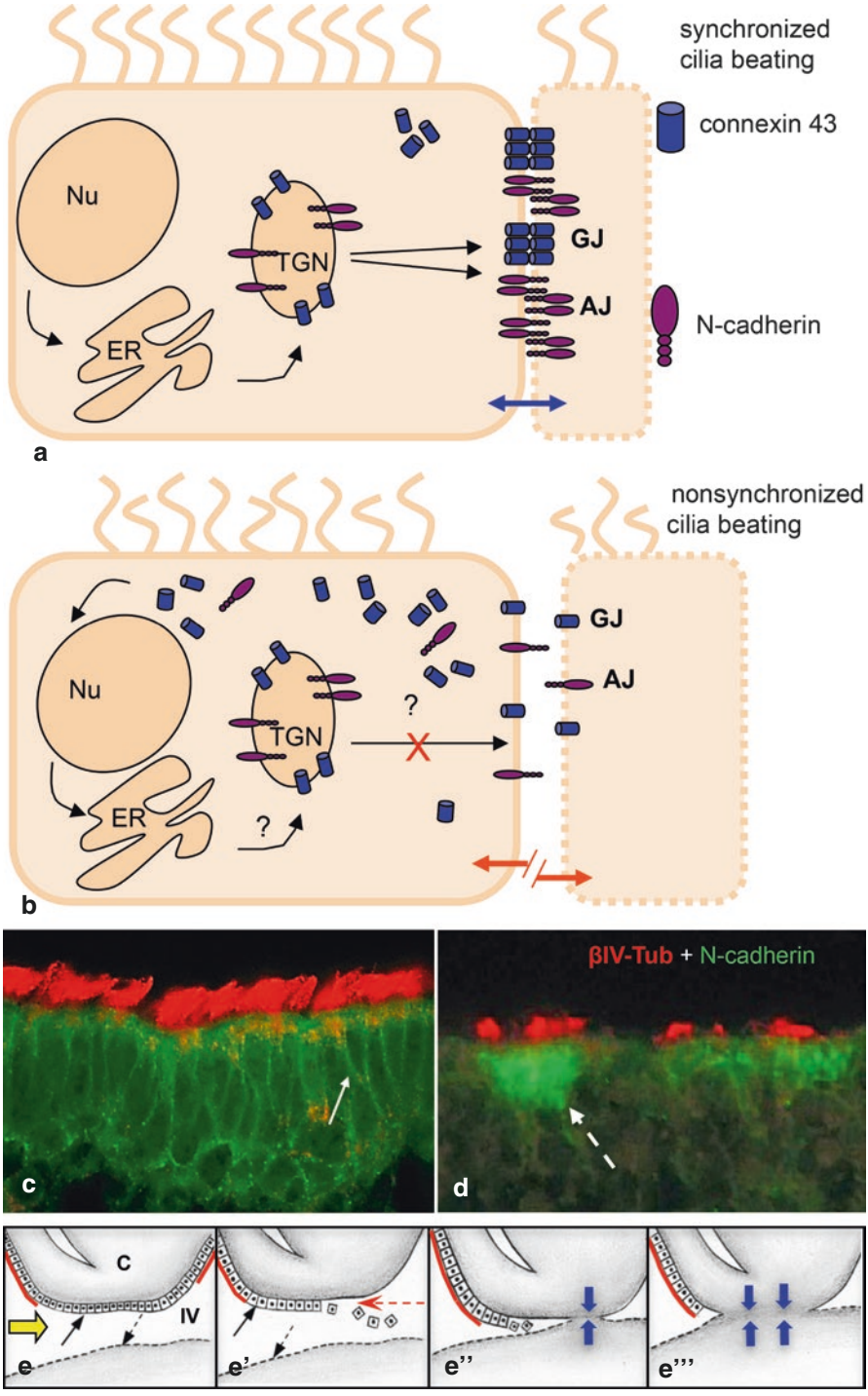

synchronized
cilia beating
connexin 43
Nu
TGN
GJ
AJ
ER
N-cadherin
a
nonsynchronized
cilia beating
GJ
AJ
Nu
?
TGN
ER
?
b
βIV-Tub + N-cadherin
c
d
c
IV
e
e'
e''
e'''

Fig. 1.3 Proposed mechanisms underlying ependymal denudation and abnormal CSF flow in the Sylvius aqueduct of spina bifida aperta (SBA) patients. The ependyma of the Sylvius aqueduct (SA) of control foetuses display a normal expression and a normal transport to the plasma membrane of the junction proteins N-cadherin and connexin 43 (**a**). This results in normal gap (GJ) and adherent (AJ) junctions. In the SA of SBA patients, N-cadherin and connexin 43 are expressed but their transport to the plasma membrane is impaired. N-cadherin and connexin 43 are abnormally accumulated in the cytoplasm, whereas functional adherent and gap junctions fail (**b**). All together, this may induce: (i) ependymal denudation, aqueduct stenosis, and CSF obstruction; (ii) nonsynchronized cilia beating, abnormal CSF flow, and may finally contribute to (iii) hydrocephalus. ER, rough endoplasmic reticulum; Nu, cell nucleus; TGN, trans-Golgi network. (**c**, **d**) Pallium of a human hydrocephalic foetus showing zones lined by normal (**c**) or abnormal (**d**) ependyma. In areas of intact ependyma, N-cadherin is localized at the plasma membrane (**c**, full arrow). Close to the disruption front, ependymal cells displayed abnormal expression of N-cadherin (**d**, broken arrows). (**e**–**e**′′′). In *hyh* mice, disruption of the VZ lining the ventral wall of the aqueduct occurs during early foetal life (**e**, broken line). Disruption of the dorsal wall of aqueduct occurs shortly after birth (**e**′, red arrow). Then the ventral and dorsal denuded walls fuse, leading to aqueduct obliteration (**e**′′, **e**′′′, arrows) and hydrocephalus. (Source: **a**, **b** from [94]; **c**, **d** from [29]; **e**–**e**′′ modified after [103])

Nongenetic mechanisms leading to VZ disruption have to be considered also [92, 112]. In fact, lysophosphatidic acid, a blood-borne factor found in intraventricular haemorrhages, binds to receptors expressed by the VZ cells resulting in abnormal N-cadherin trafficking, VZ disruption, and hydrocephalus [112]. The vascular endothelial growth factor is elevated in the CSF of patients with hydrocephalus, and when administered into the CSF of normal rats, it causes alterations of adherent junctions, ependyma disruption, and hydrocephalus [92]. Thus, the possibility that signals from the hydrocephalic CSF may contribute, or even trigger VZ disruption, has to be kept in mind. Furthermore, it should be kept in mind that foetal CSF is the internal milieu of NSC [42]. Interestingly, the CSF of hydrocephalic HTx rats has an abnormal protein composition that contribute to the abnormal neurogenesis occurring in this mutant [56, 61, 62, 101].

Temporal and Spatial Programs of VZ Disruption

The process of VZ disruption has temporal and spatial patterns. The temporal program implies that disruption starts when the VZ is formed by NSC and finishes when the VZ is formed by multiciliated ependyma. In the mean time, a progressive transition from NSC to multiciliated ependyma occurs. The spatial program discloses that disruption begins in caudal regions of the ventricular system and progresses rostrally to reach the lateral ventricles [41, 74, 103]. Each of the two programs has its own outcomes.

In the temporal program, the early VZ disruption implies the loss of NSC and abnormal neurogenesis, while the late VZ disruption results in the loss of multiciliated ependyma and alterations in the laminar flow of CSF and hydrocephalus [29, 94]. In the *hyh* mutant mouse, the program is turned on at E12 and turned off by the end of the second postnatal week [41, 74, 103]. In the HTx mutant rat, disruption in the telencephalon starts at E19 and finishes at the first postnatal week [29].

In hydrocephalic foetuses, disruption of the VZ in the telencephalon has been shown as early as 16 GW [21, 29].

In the spatial program, the disruption of the VZ of the SA implies aqueduct stenosis/obliteration, alteration of the laminar, and bulk flow of CSF and hydrocephalus. At variance, the disruption of the VZ of the telencephalon leads to abnormal neurogenesis [29, 83].

With the years and based on solid evidence, we have progressively come to the conclusion that *foetal onset hydrocephalus and abnormal neurogenesis are two inseparable phenomena, because they are linked at the etiological level.*

In the pathophysiologic programs of VZ disruption, the loss of NSC and ependyma occurs in *specific regions* of the SA and ventricular walls, and also at *specific stages* of brain development. This explains why only certain brain structures have an abnormal development, which in turn results in a specific neurological impairment.

Pathophysiology of Foetal Onset Hydrocephalus

The Complex Cell Organization of the Walls of the Sylvius Aqueduct

The walls of the Sylvius aqueduct of wild-type *hyh* mice are formed by several populations of ependymal cells [103]. Interestingly, in mutant hydrocephalic *hyh* mice, some of these ependymal populations undergo proliferation, others are resistant to denudation whereas others denude [4, 74, 103]. In full-term human foetuses, the dorsal, lateral, and ventral walls of the SA three populations of ependymal cells have been described [94]. The functional significance of three ependymal populations is unclear. However, in spina bifida aperta foetuses, there seems to be an association between ependymal lineages of SA and the observed SA pathology. The ependymal cells lining the ventral wall display a normal subcellular distribution of N-cadherin and connexin 43; these cells do not detach. At variance, the ependymal cells of the lateral SA walls display an abnormal intracellular location of junction proteins and are likely to undergo denudation. The formation of large rosettes is mostly associated to this ependyma [94].

Ventricular Zone Disruption in the Sylvius Aqueduct, Aqueduct Stenosis/Obliteration, and Noncommunicating Hydrocephalus

In the *hyh* mouse, a programmed disruption of the VZ of the ventral wall of the SA starts early in foetal life (E12.5) and *precedes* the onset of a moderate communicating hydrocephalus. The loss of the ependyma of the dorsal wall of the SA occurring shortly after birth leads to fusion of the denuded ventral and dorsal walls of SA, resulting in aqueduct obliteration and severe hydrocephalus (Fig. 1.3e–e‴) [41, 74, 103]. The phenomenon of VZ denudation associated with the onset of hydrocephalus has also been found in other mutant mice [38, 48, 52, 65, 77].

In human hydrocephalic foetuses, ependymal denudation of SA precedes and probably triggers the onset of hydrocephalus [21, 72, 94]. It can be postulated, on solid grounds, that a primary alteration of the VZ of the aqueduct due to various genetic defects triggers the onset of congenital hydrocephalus.

Ventricular Zone Disruption in the Sylvius Aqueduct, Loss of Multiciliated Ependyma and Communicating Hydrocephalus

In full-term human foetuses and in the perinatal period of mice the SA is mostly lined by multiciliated ependymal cells [94, 103]. The disruption occurring in this period in hydrocephalic humans and mutant mice implies the loss of multiciliated ependyma. Prior to denudation, the abnormal ependymal cells display abnormalities in the amount and subcellular distribution of N-cadherin and connexin 43 (Fig. 1.3) [94]. Since connexin 43 and N-cadherin co-assemble during their traffic to the plasma membrane [104], the abnormal formation of adherent junctions would also result in abnormal gap junctions. Thus, defects of adherent junctions between ependymal cells in hydrocephalic foetuses could alter gap junction-dependent ependymal physiology prior to, or in the absence of, ependymal disruption. An alteration of the CSF laminar flow through the SA of human hydrocephalic foetuses could be envisaged, even if denudation is confined to small areas of the aqueduct wall and hydrocephalus courses with a patent aqueduct (Fig. 1.3a, b). This could be part of the mechanism resulting in a communicating hydrocephalus.

At Late Gestational Stages, the Disruption in the Ventricular Zone of the Telencephalon Leads to the Loss of Multiciliated Cells and Likely Alterations in the Laminar CSF Flow

The disruption wave starting in the fourth ventricle, after a few days, reaches the telencephalon; then it continues along the walls of the lateral ventricles following a fixed route but avoiding certain discrete regions that are disruption resistant. This phenomenon occurs in certain mutant animals [41, 103] and part or most of it also occurs in human hydrocephalic foetuses [21, 29] and in premature hydrocephalic foetuses with intraventricular haemorrhage [57].

Ciliary beating of ependymal cells is responsible, at least in part, for the laminar flow of CSF occurring on the ventricular surface (see above). Long ago, Worthington and Cathcart [109] concluded that in humans, small areas of ependymal injury and ciliary destruction may affect CSF flow far beyond the region of local damage. During the third trimester of gestation, VZ disruption occurring in hydrocephalic foetuses and in cases with posthaemorrhagic hydrocephalus leaves large areas of the ventricular walls denuded [21, 29, 57]. It seems likely that these local disturbances may impair laminar CSF flow and contribute to the development of hydrocephalus.

Abnormal Neurogenesis Linked to Foetal Onset Hydrocephalus

Disruption of the Ventricular Zone of the Telencephalon Is Associated to Abnormal Neurogenesis

In human hydrocephalic foetuses [21, 29], premature infants with posthaemorrhagic hydrocephalus [57], the HTx rat [29] and the *hyh* mouse [23], the VZ disruption results in two neuropathological events: formation of periventricular heterotopias and translocation of NSC/NPC to the CSF (Fig. 1.4a–d).

At regions of disruption where NSC have been lost, the neuroblasts generated in the SVZ no longer have the structural scaffold to migrate and consequently accumulate in periventricular areas forming periventricular heterotopias. In human hydrocephalic foetuses, periventricular heterotopias have been found in young (21 GW) and full-term (40 GW) foetuses, indicating that they were formed early in development and had remained in situ until the end of foetal life and, probably, after birth (Fig. 1.4a, b). Interestingly, a 2-month-old child with a disrupted VZ carried periventricular heterotopias [23]. Humans with disruption in the VZ of the telencephalon carry periventricular heterotopias primarily composed of later-born neurons [23]. Periventricular heterotopias behave as epileptogenic foci [30]. This may explain why 6–30% of hydrocephalic children, including the present case, develop epilepsy that is not solved by CSF drainage surgery [72, 89].

The Cerebrospinal Fluid Is the Main Fate of the Disrupting NSC/NPC

In hydrocephalic human foetuses [21, 29] and premature infants with posthaemorrhagic hydrocephalus [49], NSC/NPC reach the ventricle at sites of VZ disruption and can be collected from the CSF. Furthermore, cells collected from CSF of two SBA foetuses develop into neurospheres [83].

hydrocephalic foetus, 40 GW, with a large denuded area covered by a layer of glial fibrillary acidic protein (GFAP) positive astrocytes. Periventricular heterotopias (PH) are associated with disruption of the VZ. (**c**) In the HTx rat, disruption of the VZ results in shedding of proliferative neural progenitor cells into the CSF, as shown by injection of BrdU in living animals and tracking the BrdU+ cells in tissue sections (**c**, arrow) and CSF cell pellets (**d**). βIII-tubulin+ or nestin+ cells are present in the cell pellet (**d**). (**e**, **f**) Under proper culture conditions, cells grow forming neurospheres displaying a similar junction pathology than hydrocephalic living animals. In neurospheres from non-affected HTx rat, N-cadherin is located at the plasma membrane (**e**); in neurospheres from hydrocephalic CSF N-cadherin accumulates in the cytoplasm (**f**). (**g**) Line drawing depicting the pathology of ventricular zone (VZ). Whereas disruption in the aqueduct of Sylvius leads to hydrocephalus, VZ disruption in the telencephalon results in abnormal neurogenesis. A cell junction pathology appears to be a final common pathway of multiple genetic and environmental factors that finally result in the disruption of the VZ. (Source: **a**–**g** from [29])

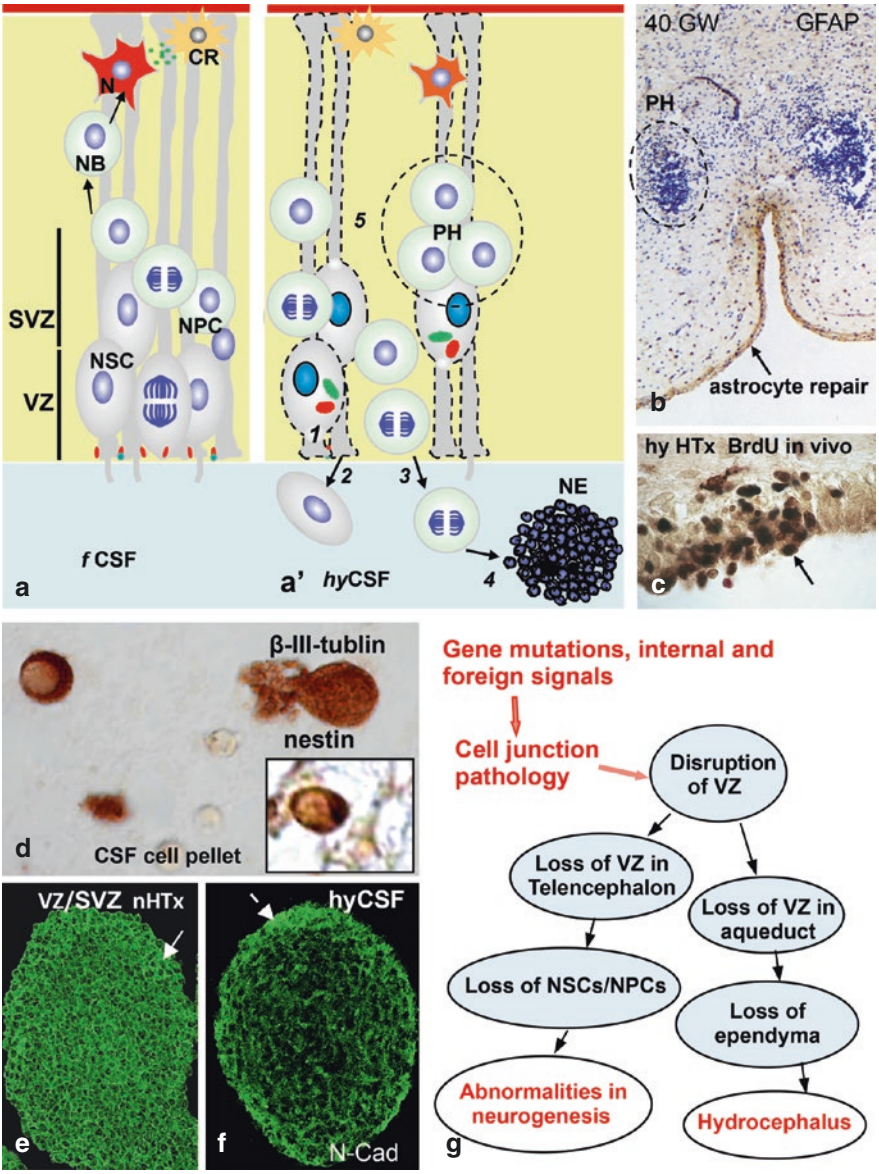


Fig. 1.4 Neuropathological events associated to the disruption of the ventricular zone in the telencephalon**.** (**a**, **a′**) Line drawings depicting the pathology. (**a**) Neural stem cells (NSC) in the ventricular zone (VZ) proliferate to raise proliferative neural progenitor cells (NPC), which migrate as neuroblasts (NB) along radial processes of NSC and differentiate into neurons (N). NSC are joined by adherent and gap junctions. CR, Cajal-Retzius cell; fCSF, foetal cerebrospinal fluid; SVZ, subventricular zone. (**a′**) Disruption of the VZ results in displacement of NSC [1] and NPC [2] into the CSF [3]. These cells can be collected from the CSF of hydrocephalic rats and cultured. They develop abnormal neurospheres [4]. The absence of the scaffold provided by NSC results in arrested neuroblasts that form periventricular heterotopias (PH) [5]. (**b**) Telencephalon of a human

In the hydrocephalic HTx rat, proliferative NPC from the SVZ reach the ventricle through the sites of VZ disruption and can be collected from the CSF. Nestin+ NSC from the VZ also appear to reach the CSF (Fig. 1.1c, d). When processed for the neurosphere assay, the cells collected from CSF proliferate and become assembled again through adherent junctions to form neurospheres. After 2 days in culture, the neurospheres start to express an adherent junction pathology (Fig. 1.4e, f) and become disrupted, mirroring the pathology of NSC in the VZ of the living hyHTx. This finding strongly indicates that a genetic defect and not epigenetic factors, such as increased CSF pressure or changes of CSF composition, underlies the disruption phenomenon.

The findings discussed indicate that NSC and NPC collected from the CSF of hydrocephalic patients can be used to investigate cell and molecular alterations underlying the disease. Thus, the inability to obtain human brain biopsies for diagnostic and research reasons may be overcome.

In brief, the evidence discussed in the present chapter identifies a new mechanism underlying the abnormal neurogenesis associated to foetal-onset hydrocephalus (Fig. 1.4g). A cell junction pathology of NSC is associated to the disruption of the VZ, the formation of periventricular heterotopias, and the abnormal translocation of NSC and NPC to the foetal CSF. The outcomes of these abnormalities continue to the end of foetal life and most likely during postnatal life. These abnormalities could explain the neurological impairments, such as epilepsy, of children born with hydrocephalus. Furthermore, the new evidence also provides the basis for the use of the neurosphere assay for diagnosis and cell therapy [29, 83]. We agree with Del Bigio [19] and Williams et al. [105] that "better treatment of hydrocephalus and the associated neurological impairment will come from a better understanding of the biological basis of the brain abnormalities in hydrocephalus."

Repair Mechanisms of the Disrupted Ventricular Zone

In the *hyh* mouse, the pathophysiologic program leading to hydrocephalus includes a repairing stage in which the missing VZ is replaced by a layer of astrocytes forming a new interface between the CSF and the brain parenchyma (Fig. 1.5a–c) [74, 79, 103]. This unique astrocyte layer prevents the NPC still present in the SVZ from being displaced into the ventricle. This response occurs shortly after VZ disruption and takes weeks to complete [74, 79]. This phenomenon has also been described in the hydrocephalic HTx rat [29] and the human hydrocephalic foetuses [21, 29, 57, 87, 94].

The astrocytes re-populating the denuded areas are different from astrocytes of the normal brain parenchyma and from reactive astrocytes found after brain injury. They share several cytological features with multiciliated ependyma and similar para-cellular and intra-cellular routes of transport of cargo molecules moving between CSF, the subependymal neuropile and the pericapillary space (Fig. 1.5a–c) [79]. How do astrocytes arriving at the denuded ventricular surface become arranged into a compact cell layer? In the *hyh* mice, the numerous interdigitations between

the cell bodies of astrocytes and the dense network formed by their processes might explain the stability of this newly formed cell layer (Fig. 1.5b, c) [79]. What are the signals mediating this response? In *hyh* mice and human hydrocephalic foetuses, VZ disruption takes place at prenatal stages previous to a detectable hydrocephalus. Therefore, intraventricular pressure or expanding ventricles cannot be considered responsible for the denudation of the VZ or its repairing by astrocytes [79].

In *hyh* mice, the periventricular astrocyte reaction appears at stages when ventriculomegaly is starting to develop. The most robust astrocyte layer occurs in the denuded floor of the fourth ventricle, a cavity displaying a minor dilatation [79]. What are the physiopathological consequences for the brain of the assembly of a compact layer of astrocytes replacing the lost ependyma? This is an important question open to investigation. Still, there are already some clues. Astrocytes replacing the denuded ependyma have a high expression of AQP4 and a high endocytosis and transcytosis activity, suggesting they function as a new CSF–brain interphase involved in water and solute transport, contributing to re-establish some of the functions of the lost ependyma [79].

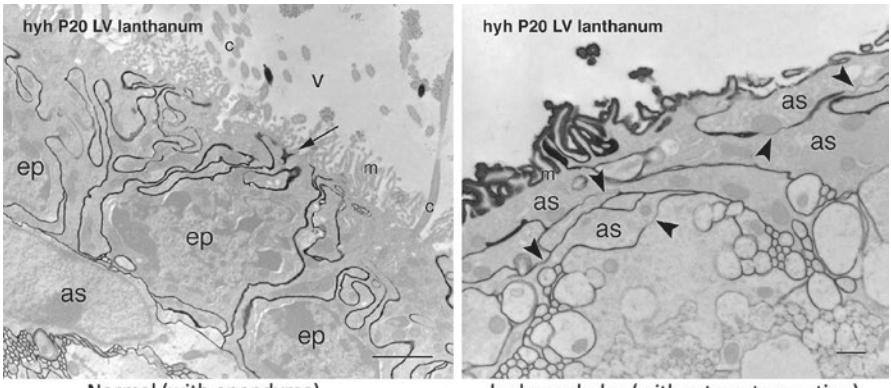
Interestingly, the disruption of the VZ that occurs in foetal life of the hydrocephalic HTx rat and the repairing astrocyte mechanisms occurring postnatally is followed by a second disruption process, this time affecting the astroglial layer. The outcome of this new disruption is the massive translocation of neurons into the ventricle [73]. This second and devastating disruption process observed in 1-month-old rats could be part of the mechanism leading to death.

Cell Therapy in the Horizon

Once establishing that foetal-onset hydrocephalus and abnormal neurogenesis are two inseparable phenomena turned on by a cell junction pathology first affecting NSC/NPC and later the multiciliated ependyma; the grafting of stem cells into hydrocephalic foetuses appears as a valid therapeutic task to repair the VZ disruption and its outcomes.

Growing evidence has shown that stem cell transplantation represents a great opportunity for the treatment of many neurological diseases. Stem cells used for transplantation into the central nervous system (CNS) include mesenchymal stem cells (MSC) [84], NSC [3, 9], and NPC [70, 110]. In most of the early investigations, the stem cells were grafted in the vicinity of the injured or altered neural tissue. However, delivery of stem cells into the CSF is emerging as an alternative, particularly for those diseases with a broad distribution in the central nervous system [3, 67, 75, 110]. A key question whether the hydrocephalic CSF would be a friendly medium to host grafted NSC has been recently solved. When neurospheres obtained from non-affected HTx rats are further cultured in the presence of CSF from hydrocephalic HTx rats, neural stem cells differentiate into neurons, astrocytes, and ependyma [33].

On-going experiments in our laboratory grafting normal neurospheres into the lateral ventricle of hydrocephalic HTx rats has shown that 48 hs after


hyh P20 LV lanthanum
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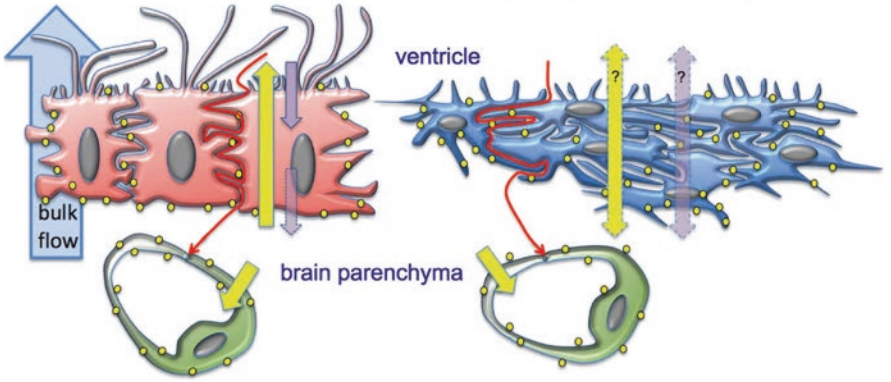
Normal (with ependyma)
hydrocephalus (with astrocyte reaction)


ventricle
bulk
flow
brain parenchyma

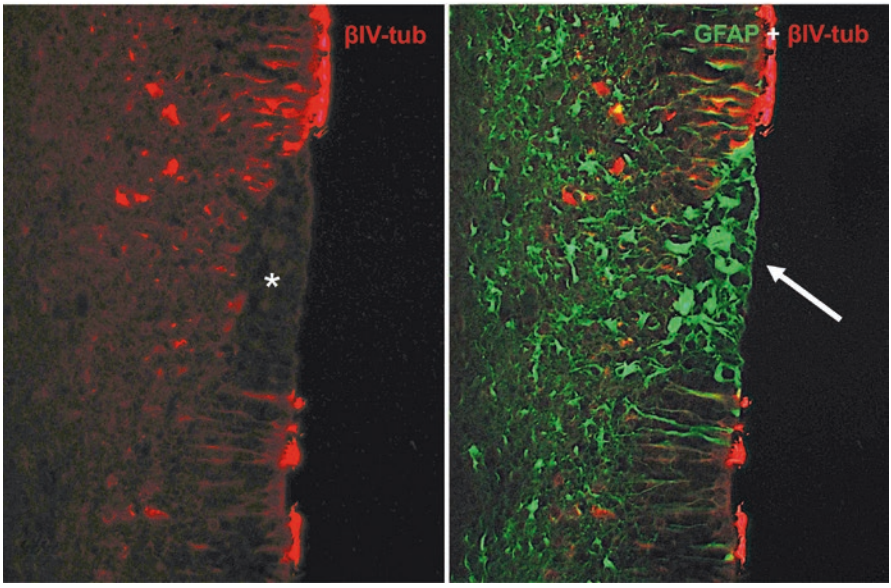

βIV-tub
GFAP + βIV-tub

Fig. 1.5 (**a**) Lanthanum nitrate applied into the lateral ventricle of a P20 *hyh* mouse penetrates from the lateral ventricle (V, arrow) toward the brain parenchyma through the winding extracellular spaces of the denudation-resistant, ciliated ependyma (ep). (**b**) In the astrocyte layer (as) lining the denuded ventricular surface of a *hyh* mouse, the tracer penetrates through the extracellular spaces and bypasses the gap junctions joining the astrocytes (arrowheads). **c**, cilia; m, microvilli. (**c**) Representation of the transcellular and paracellular transport mechanisms that would operate at the ependyma and at the layer of repairing astrocytes. In the ependymal cells of wt mice (left), most aquaporin 4 (yellow dots) is located at the basolateral domains, suggesting that the ependyma transports water from the brain parenchyma (bottom) toward the ventricular CSF (upper) (thick arrow across the ependyma). There is pinocytosis and transcytosis directed in the opposite direction through this barrier (purple arrow). In *hyh* mouse (right), a layer of astrocytes covering the denuded surface express aquaporin 4 throughout the cell body and processes (yellow dots) and could be involved in water transport from or to the CSF (double-headed yellow arrow). Pinocytosis in the astrocytes would also operate in both directions (double-head purple arrows). The ependymal and the astrocyte barriers would transport molecules from the CSF to the brain parenchyma through a paracellular route (winding red arrows). (**d**, **d′**). Disruption process affecting the VZ and SVZ of preterm neonates with intraventricular hemorrhage. The VZ formed by multiciliated cells also undergoes disruption (**d**, asterisk); the disrupted foci are sealed by a layer of GFAP+ astrocytes (**d′**, arrow). (Source: **a**–**c** from [79]; **d**, **d′** from [57])

transplantation, the grafted NSC moves *selectively* to the area devoid of VZ, proliferate, and differentiate into patches of multiciliated ependyma; a second subpopulation move into the cerebral cortex. According to our current investigations, it seems likely that the new multiciliated ependyma formed after NSC grafting would help the laminar flow of CSF and, consequently, attenuate the hydrocephalus condition. If NSC grafting results in a functional recovery of the neurological deficit of the rats born with hydrocephalus is under research.

Toward the frontier of the bed side The isolation and expansion of NSC of human origin are crucial for the successful development of cell therapy approaches in human brain diseases. A relevant step forward has been achieved by scientists of the Neuroscience Center of Lund (Sweden) who developed an immortal neural stem cell line and have standardized a protocol to obtain neurospheres from foetal striatum-derived neural stem [10, 63]. An additional key point to consider is the time and opportunity when NSC should be transplanted. It seems reasonable to suggest that NSC grafting should be performed shortly after the disruption process of the VZ had been turned on. In human hydrocephalic foetuses, VZ disruption starts at about the 16th GW and continues throughout the second and third trimester of pregnancy (see above). The opportunity for transplantation may be the foetal surgery performed to repair neural tube defects, such as spina bifida aperta, that is performed within a well-defined gestational period (19th–25th GW) [1]. It may be hoped that grafting of stem cells into the hydrocephalic brain would result in the repopulation of the disrupted areas of the VZ and/or the generation of a protective microenvironment to diminish/prevent the outcomes of VZ disruption. (Fig. 1.6).

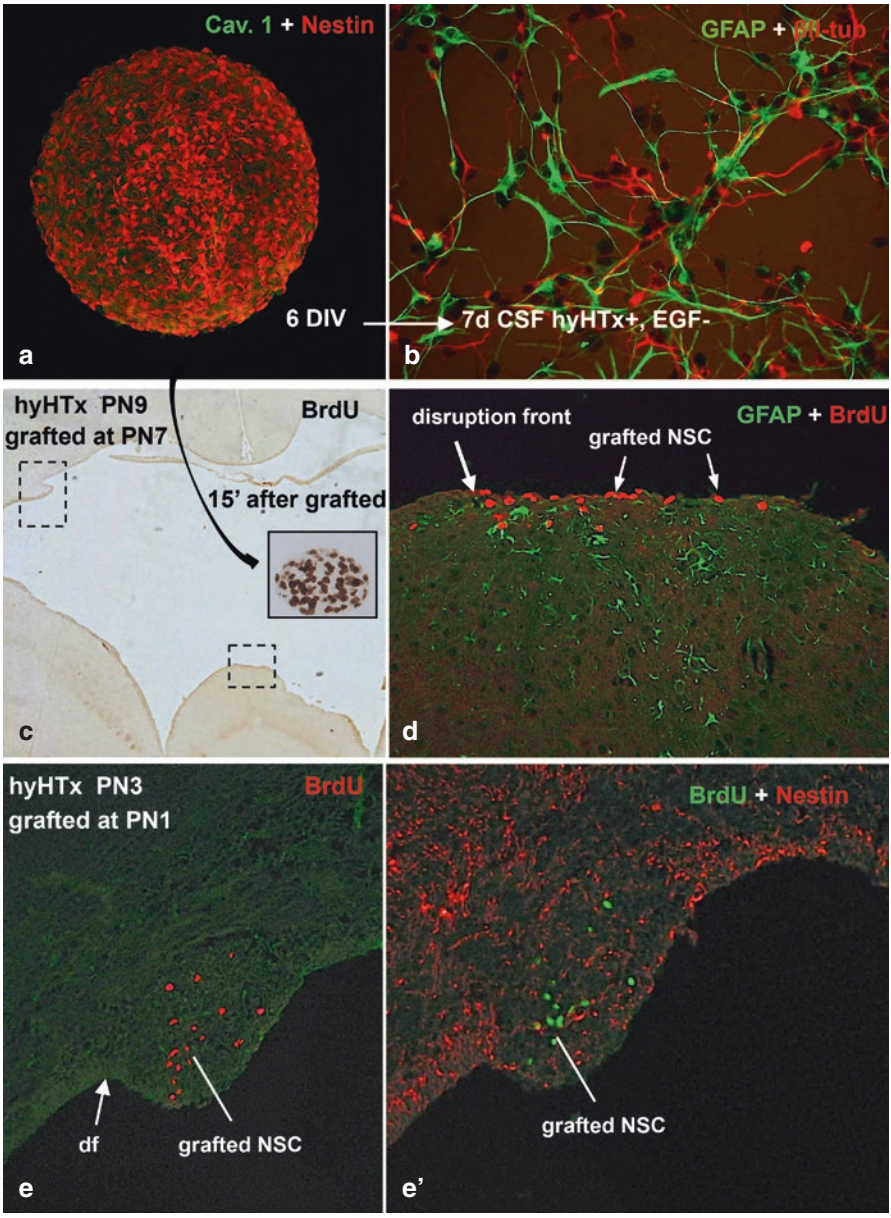


Fig. 1.6 Neural stem cells grafted into the cerebrospinal fluid (CSF) of a hydrocephalic HTx rat move selectively to the disrupted areas of the ventricular zone (VZ). (**a**) Neurosphere after 6 days in culture immunostained for nestin. (**b**) In the presence of hydrocephalic CSF and devoid of epidermal growth factor, neural stem cells differentiate into βIII-tubulin+ neurons and GFAP+ astrocytes. (**c**–**e′**) Grafting of neurospheres obtained from a non-affected HTx rat on postnatal day 1 (PN1) to PN1 and PN7 hydrocephalic HTx rats. Grafted neurospheres were labelled with BrdU during the last 24 h in culture. (**c**) 15 min after grafting neurospheres remain proliferative and free inside the dilated lateral ventricles *(inset)*. (**d**–**e′**) Two days after grafting, neurospheres disassemble and the NSC move selectively to the disrupted areas of the VZ. df, disruption front. (Source: **a**–**e′** from [83])

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