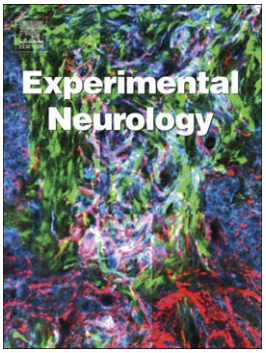

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A Balanced View of the Cerebrospinal Fluid Composition and Functions:

Focus on Adult Humans

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Abstract

In this review, a companion piece to our recent examination of choroid plexus (CP), the organ that secretes the cerebrospinal fluid (CSF), we focus on recent information in the context of reliable older data concerning the composition and functions of adult human CSF. To accomplish this, we define CSF, examine the methodology employed in studying the CSF focusing on ideal or near ideal experiments and discuss the pros and cons of several widely used analogical descriptions of the CSF including: the CSF as the "third circulation," the CSF as a "nourishing liquor," the similarities of the CSF/choroid plexus to the glomerular filtrate/ kidney and finally the CSF circulation as part of the "glymphatic system." We also consider the close interrelationship between the CSF and extracellular space of brain through gap junctions and the paucity of data suggesting that the cerebral capillaries secrete a CSF-like fluid. Recently human CSF has been shown to be in dynamic flux with heart-beat, posture and especially respiration. Functionally, the CSF provides buoyancy, nourishment (e.g., vitamins) and endogenous waste product removal for brain by bulk flow into the venous (arachnoid villi and nerve roots) and lymphatic (nasal) systems, and by carrier-mediated reabsorptive transport systems in CP. The CSF also presents many exogenous compounds to CP for metabolism or removal, indirectly cleansing the extracellular space of brain (e.g., of xenobiotics like penicillin). The CSF also carries hormones (e.g., leptin) from blood via CP or synthesized in CP (e.g., IGF-2) to brain. In summary the CP/CSF, the third circulation, performs many functions comparable to the kidney including nourishing the brain and contributing to a stable internal milieu for brain. These tasks are essential to normal adult brain functioning.

Keywords

Brain extracellular space; CSF circulation; water movement; blood-CSF barrier; blood-brain barrier; glymphatic system; choroidal reabsorptive transport; cerebrospinal fluid peptides;trans-ependymal exchange; trans-pial exchange; neurogenesis; CSF streaming; primary ciliary dyskinesia; hydrocephalus; glia; immunomodulation

Abbreviations (for footnote)

AA, ascorbic acid

Aβ, amyloid beta

AE-2, anion exchanger-2

AQP, aquaporin

BBB, blood-brain barrier

BCSFB, blood-CSF barrier

BDNF, brain derived neurotrophic factor

CP, choroid plexus

DPH, diphenhydramine

ECSB, extracellular space of brain

GLUT-1, glucose transporter

5-HIAA, 5-hydroxyindole acetic acid

HVA, homovannilic acid

IFN-1, interferon-1

IGF, insulin-like growth factor

IsoA, iso-ascorbic acid

K_T, half saturation constant

NBCe1, sodium bicarbonate e1 cotransporter (electrogenic)

NBCn1, sodium bicarbonate n1cotransporter (neuronal expression)

NO, nitric oxide

OAT, organic acid transporter

P, permeability constant

PCD, primary ciliary dyskinesia

SVCT, sodium vitamin C transporter

Contents

Introduction

In this review, we focus on recent information in the context of reliable established data concerning the composition and manifold functions of adult human cerebrospinal fluid (CSF). This review is a companion to our recent treatise on the structure and function of the choroid plexus (CP) that secretes the CSF (Spector et al., 2015). We will not discuss children less than two years of age because the functions, flow and reabsorption of CSF in their development are not yet fully studied (Bateman and Brown, 2012; Whish et al. 2015). Also, due to the length consideration of this review, we do notcomprehensively treat spinal cord-CSF relationships and associated clinical problems,e.g. syrinx;however, in this regard it is important to acknowledge that Koyanagi et al. (2010), and Ushewokunze et al. (2010), respectively, have thoroughly analyzedpathogenetic and surgical aspects of syrinomyelia.To understand properly the composition and functions of CSF, we carefully define CSF (Table 1), examine the significant methodologic problems in studying CSF, exemplify and focus on ideal or nearly ideal experiments among hundreds performed, and explicate the differences between CSF formation by CP and the enormous H_2Oexchange fluxes inCNS (Davson et al., 1987: Bateman and Brown, 2012; Spector et al., 2015).

As a means of exploring the numerous functions of CSF we employ, in part, an historical approach discussing several widely used analogical descriptions of the CSF as the “third circulation” (Cushing, 1925), the CSF as a “nourishing liquor,” similarities between the CSF/CP on the one handand the renal glomerular filtrate/kidney tubules on the other, and finally the CSF circulation as part of the “glymphatic system” (Iliff et al., 2012; 2013; Xie et al., 2013)(an analogy with the lymphatic system).

In the Introduction, we briefly introduce these analogical descriptions.In the body of the paper is a discussion of the issues in detail, as well as the possibility of extra-CP CSF formation, and molecular exchange between the CSF and extracellular space of brain [ECSB] (also called interstitial fluid). Finally, throughout the review we address certain misconceptions about the CSF and briefly mention, where appropriate, the clinical implications of recent developments in understanding CSF composition and functions. Noteworthy is the fact that the blood-CSF barrier (BCSFB) consists of the CPepithelial cells and arachnoid membrane cells joined by tight junctions that inhibit the passive flux of H_2O-soluble molecules (Spector et al., 2015). However, there is no appreciable barrier to diffusion between CSF and ECSB because the pial and ependymal linings contain gap junctions in animals and humans (Whish et al., 2015).In humans, the CSF volume is about 150 ml with ~20% residing in the lateral, third and fourth ventricles. The volume of the human ECSB is also ~150 ml (Davson and Segal, 1996). The ECSB,comprising ~15% of brain volume,is a tortuous conduit among cells that slows down simple diffusion by ~50% in part due to large matrix molecules (viscous) in the ECSB (Hladky and Barrand, 2014). Since cortical ECSBdecreases progressively as brain size becomes smaller (Greenberg et al., 1965), caution is in order when comparingmurine vs. human ISF-CSF dynamics.For a schematic diagram of the relationshipbetween ventricular CSF, ependyma and ECSB, the reader is referred to Fig. 2 in the article by Spector and Johanson, 1989.

For centuries, the CSF has fascinated philosophers, and more recently anatomists, physiologists, biochemists and neurologists who have made progress in defining and understanding the CSF. With the development of anesthesia, neurosurgeons became very interested in CSF. Harvey Cushing synthesized the extant information in 1925 and correctly identified CP as the source of CSF, the bulk flow of CSF through the ventricular system, and the

exit of CSF through the foramina of Luschka and Magendie (fourth ventricle) into the subarachnoid space with subsequent absorption into venous blood. He termed this process the "third circulation," the first being that of the blood; and the second, the lymph. Cushing (1925) recognized the important role of the CSF in providing buoyancy for the ~1.2 kg human brain, and so, by the Archimedes principle, operationally only "weighing" ~45 g.

Around the same time, others (Stern and Gautier, 1921; 1922; 1923) proposed the CSF asa "nourishing liquor." This concept now has substantial merit because CSF contains certain essential substances including several micronutrients (e.g., vitamin C, folate) (Spector, 2014; Spector and Johanson, 2014), ions, peptides, proteins and ~90 varieties of small RNA not found in plasma whose function(s) awaitdetermination(Gallego et al., 2012).These substances penetrate into brain from CSF, and are essential for brain health (Table 1). Of course macronutrients (e.g., glucose, amino acids, and lactate) and many micronutrients, hormones, vitamins and minerals are transported from blood directly into brain by specialized mechanisms in the brain capillaries (Davson and Segal, 1996). Brain capillaries are joined by very tight junctions and are the locus of the blood-brain barrier (BBB) (Whish et al., 2015). Thus, except where there are specialized systems that allow entry (and exit), both the BBB and BCSFB isolate the mammalian brain and CSF from many H_2O-soluble molecules (including drugs) in the blood. Molecular transfer of drugs from blood into ECSB and the CSF, respectively, through the BBB and ECSB depends on several important factors: the size, charge, lipid solubility and plasma protein binding of the molecule; and secondly, the affinityof the molecule, if any, for influx and/or efflux transporters at the BBB and /or BCSF barrier as described below (Spector 2009; 2010; Spector and Johanson, 2006).

Several pioneering investigators (Pappenheimer et al., 1961; 1962) introduced the technique of ventriculo-cisternal perfusion.They noted the resemblance of the kidney tubules and CP, respectively, in handling many molecules between glomerular filtrate and blood, and between blood and CSF. This led to the so-called "sink" analogy discussed below. The largely protein-free glomerular filtrateand CSF are dissimilar in that the glomerular filtrateis formed by a *passive* process and the CSF *actively* secreted by CP (Davson et al., 1987; Spector et al., 2015). In many respects, however, the CP and kidney behave similarly. For example, in humans, from the glomerular filtrate(~200 l/d which contains ~1 g of ascorbic acid [AA]), the ~700 g human kidneys (renal tubules) *reabsorb* >99 % of the AA, by an active transport mechanism termed the sodium-dependent vitamin C transport system-1 (SVCT-1). The ~2 g human CP, through which flows ~12 l/dof blood containing ~45 mg of AA, extracts and *secretes*>50% of the blood AA into CSF by a similar mechanism (SVCT-2) (Spector and Johanson, 2006; 2014). This explains the ~4 times higher AA concentration in CSF than plasma. In the *reabsorbing*renal tubules, the SVCT-1 is on luminal side; in the *secreting*CP the SVCT-2 is on the abluminal or basal side (Spector and Johanson, 2006; 2014).

On the other hand, the renal tubules and CP epithelial cells transport manyunneeded endogenous (e.g., inactive metabolites) and exogenous substances (e.g., penicillin) out of blood into urine, and out of CSF into blood, respectively (Davson et al., 1987). For example, the main system that transportsplasma penicillin into urine (via tubules), and CSF penicillin into blood (by CP), is the organic acid transporter-3 (OAT-3) (Spector, 2010; Spector et al., 2015).

In the past two decades, one group has repeatedly suggested that the CSF in not secreted by the CP and does not flow down the aqueduct of Sylvius (Oreskovic and Klarica, 2010; 2014a; 2014b). They suggest that CSF is secreted by and reabsorbed by the cerebral capillaries. This

theory is not consistent with the older data reviewed in the magisterial reviews by Davson and collaborators (1987;1996), and with more recent data (Mokgokong et al., 2014; Spector et al.; 2015). In reviews, this view of the BBB secreting true CSF has been thoroughly debunked by Brinker et al. (2014); Bateman and Brown (2012); Hladky and Barrand (2014); Spector et al. (2015) and by numerous neurosurgeons (Tisell, 2005; Warf et al., 2012; Bateman and Siddique, 2014). These erroneous views are due to lack of understanding of well-established CP functioning, questionable protocols in experimental work in heavily-instrumented anesthetized cats, and misinterpretation about CSF secretion vs. H_2O exchanges among blood, brain and CSF (that are >100 times the rate of CSF secretion in both animals and humans) (Bateman and Brown, 2012; Hladky and Barrand, 2014; Spector et al., 2015). Moreover, there are evidently no mechanisms in cerebral capillaries in vivo (not withstanding heroic efforts to find them) to secrete CSF-like fluid into the ECSB with subsequent flow into the CSF (Mokgokong et al., 2014). These issues are discussed in detail below.

For over a century, there has beenclear recognition of free exchange of molecules mainly by diffusion,across ependymal and pial interfaces on the one hand, and flowing ventricular and subarachnoid CSF on the other. This diffusion occurs through permeable "gap" junctions (Whish et al. 2015). However, there is now convincing human data that CSF flow is not unidirectionally-smooth like a river but there is bidirectional flow (oscillatory) through the aqueduct of Sylvius with the heart-beat, posture and especially inspiration (Table 2) (Dreha-Kulaczewski et al., 2015). On balance, the *net* flow of CSF through the ventriculo-subarachnoid system is, on average, ~0.4 ml/min (humans) with more CSF secreted at night (Nilsson et al., 1992; 1994; Spector et al., 2015). Therefore, the exchange of many substances between CSF and ECSB depends on both

diffusion through gap junctions in the pia/ependyma linings and the to-and-fro motion of the CSFmixing (Table 2).

Recently, the notion of a "glymphatic system" has been proposed in mice and rats and extrapolated to humans based on heavily-instrumented, anesthetized mouse and rat studies (Iliff et al., 2012; 2013). Theirworking model is that molecules do not mainly diffuse bidirectionally between ECSB and CSF via the ependyma/pia by way of the gap junctions; instead, most H_2O-soluble molecules leave CSF spaces and flow into the ECS of brain predominantly down arterial sheaths (possibly the Virchow-Robin spaces) into ECSB and then, after mixing with ECSB fluids, flow out of brain via cerebral veins into subarachnoid CSF. This concept is notstrongly supported by evidence to date.At most,it is a minor pathway, especially across the ependyma where there are no large penetrating vessels and where substantial CSF-ependymal exchange occurs (Hammarstrom, 1966; Spector 1981; Spector and Lorenzo, 1974: Proescholdt et al. 2000; Whish et al.l, 2015).

Finally, a recent study in mice provides exciting evidence that *aged* brain transfers signals via CSF that in turn *inhibit* CP (presumably by a feedback mechanism) from producing maintenance peptides and proteins for brain via CSF distribution (Baruch et al., 2014). Thus, there is normallycontinual bidirectional flow of'molecular information' (biochemical signals)between CP-CSF and brain, but thisis compromised in aged mice (Baruch et al., 2014). If true in humans, this is anespecially promising new finding on intra-CNS information transfer mechanisms mediated by CSF.

Definition of CSF

CSF is the secretion of CP(Johanson et al., 2008; Spector et al.,2015).A small amount of fluid, though, may come through the BBB into the ECSB by diffusion (Mokgokong et al., 2014).

CSF is principally made up of Na^+, Cl^- and HCO_3^- with lesser amounts of K^+, Mg^{++}, Ca^{++}; certain vitamins (e.g., folate, AA, thiamine and pyridoxal monophosphates); and peptides and proteins actively transferred into CSF from blood (e.g., leptin) or synthesized in CP (e.g., transthyretin, insulin-like growth factor [IGF], brain-derived neurotrophic factor [BDNF]) and then transferred into CSF (Table 1) (Spector and Johanson, 2013).

A very small quantity of plasma proteins diffuses into CSF from plasma (e.g., albumin). Unlike plasma (~7g protein/100 ml) or milky lymph (~2 g protein/100 ml), the CSF has only ~0.025 g protein/100 ml – mainly albumin. To make up for the missing negative charges on plasma proteins and the lower concentration of HCO_3^- in CSF, the Cl^- concentration is ~12 mM higher; also higher than plasma are the concentrations of Mg^{++}, AA and folate. However, CSF K^+concentration is about 2/3 that in plasma. Truly impressive are the homeostatic mechanismsin CP and glia to keep the concentrations of crucial substances stable in CSF-ECSB, e.g., for K^+(Verkhratsky and Butt, 2013). When the salt-containing CSF is secreted by CP, H_2O molecules are "dragged" along to maintain osmolality (Spector et al., 2015).

Technical issues pertaining to CSF

In evaluating experimental and clinical data on CSF composition and functions, several factors must be recognized (Table 3) as often contributing to less than ideal experimentation. First, anesthesia has very complex direct and indirect effects on cerebral blood flow, cerebral function, body temperature and CSF production. Such effects depend on the specific anesthetic, dose, and time of anesthesia (Davson et al., 1987; Hladky and Barrand, 2014). Certain drugs also have large effects on CP blood flow and/or CSF production. For example, atriopeptin (atrial natriuretic peptide) increases blood flow to CP in anesthetized rabbits but decreases or does not change CSF production. Acetazolamide on the other hand, doubles CP blood flow but *decreases*

CSF production by 55% in chloralose-anesthetized rabbits, thus uncoupling CP blood flow and CSF secretion (Schalk et al., 1992; Faraci et al., 1990). Adrenergic discharge and adrenergic agents *both decrease* CP blood flow as well asCSF production (Lindvall et al., 1979; Faraci et al., 1988).

Body temperature, blood gases (pH, pO_2, pCO_2) and plasma osmolality may affect CP blood flow and alter CSF production/flow. An excellent example of these effects is the body temperature studies by Snodgrass and Lorenzo (1972); as body temperature declined in anesthetized cats, so did CSF production.This is expected since CSF formation is directly linked to choroid epithelial metabolic rate, which decreases proportionally with temperature reduction.Another example is a recent MRI study in conscious humans demonstrating that inspiration causes marked to-and-fro motion of CSF through the aqueduct of Sylvius – more than that due to cardiac pulsations.Thus, inspiration promotes mixing in the ventricular compartment (Table 2; Dreha-Kulaczewski et al., 2015). During anesthesia, with slower and shallower breathing, this mixing effect would be minimized.

Cell culture findings are well known to be occasionally misleading. For example, brain capillaries in vivo do not normally express SVCT-2 or aquaporin 1; however, in tissue culture after several generations, they *dedifferentiate* and express substantial SVCT-2 and aquaporin 1(Spector, 2009;Verkhatasky and Butt, 2013). Instrumentation in the CNS and pressure injections (from added injectate volume) can cause inflammation and bleeding thatinterfere with normal physiology, leakage of CSF and hydrostatic pressure gradient abnormalities.

Between rodents and humans, there are profound differences in brain, not only in size, but also composition (Table 3) (Verkhratsky and Butt, 2013; Herculano-Houzel, 2014). For example, the neuronal/glial cell ratio is much larger in rodents. Protoplasmic astrocytes are 10 x

larger in humans and have an order of magnitude more functionality per astrocyte. Certain functions of the microglia are different.Moreover, the distance from the CSF to the deepest part of the brain is more than 3 xgreater in humans than rabbits. Also, the site of injection into CSF of a test agent may be critically important in regard to the brain response in translational investigations (Beard et al., 2015).

In human adults, new neuron production is limited to the dentate gyrus (<2 % /year) and striatum (<1 % /year) (Ernst et al., 2014). In rodents, there is also new neuron production in the sub-ventricular zone for transfer of interneurons via the Rostral Migratory Stream (RMS) into the olfactorybulb (Ernst et al., 2014). CSF flow linked to ciliary beating in rodent ventriclesguides nascent neuronsto olfactory target regions (Sawamoto et al., 2006). However, it is worth noting that CP histology, transport functions, CSF production rates/g and composition,from mice to humans, are remarkably similar taking brain size into account. For example, the weight of CP in rabbits is 0.020-0.025 g and ~2 g in humans; the corresponding brain weights are ~10 g and 1200g, respectively. Accordingly, for rabbits and humans, as well as rats, the CP/brain mass ratio is ~0.002.

Another important consideration for analyzing CSF is that humans are upright and rodents are not. To understand the human CSF system, gravitational forces must be taken into account. This effect of gravity is critical in some long-term missions (>6 mo in space) during which astronauts experience loss of visual acuity. In these astronauts subject tomicrogravity, there is flattening of the back of the eyeball and dilation of the CSF-containing sheath around the edematous optic nerve head (Mader et al., 2013). Due to putatively-elevated ICP in space flight (affecting ~1/2 of astronauts), the exact cause of the intracranial hypertension importantly needs

resolution (Kramer et al., 2015) due to the potentially deleterious pressure effects of CSF pressure on the eye and brain.

Whenevaluating experimental results, it is important of course to know the state of the brain and CSF at the end of the experiment. Is there inflammation or bleeding? These are crucial questions to evaluate reliability. Finally, wasthe experimentation valid,with theappropriaterandomized controls, dose-response data and time courses? Straight withdrawal of CSF (without systemic anesthesia) for CSF composition studiesdoes not suffer from these technical deficiencies. Thus, ideal ethical experimentsshould be done in normal conscious animals or humans, un-instrumented whenever possible, and with follow up of the state of subjects at the end of the investigation. There are not many near-ideal experiments but there are a few in animals (e.g.,Hammarstrom, 1966; Spector, 1981; Spector and Lorenzo, 1973; 1974; Spector et al., 1977; Proescholdt et al., 2000) and humans (Nilsson et al., 1992; 1994; Bateman and Brown, 2012; Dreha-Kulaczewski et al.,2015). Moreoversince there are significant differences between animals and humans, e.g., in the neuroanatomy of CSF drainage pathways (Coben, 1967), the findings onhydrodynamics in the formerneed validation in patients whenever possible before drawing firm conclusions about relevance to humans.

The ‘third circulation’

Despite challenge, the concept of the third circulation (Cushing, 1925) has stood the test of time. There is overwhelming evidence for the adult CP secreting CSF (Table1) that flows through the ventricles into the subarachnoid space, then over the top of the brain,withsome CSF going down to the lumbar sac. CSF exits the subarachnoid space (due to pressure-dependent bulk flow) into the blood at the arachnoid granulations;and into the lymph system through the nasal cribriform plate or via the spinal nerve roots. CSF clearance from CNS by these routesdepends

on posture, pressure differentials and pathophysiology (e.g., hydrocephalus) (Davson and Segal, 1996; Bateman and Brown, 2012; Bateman and Siddique, 2013; Hladky and Barrand, 2014).

The major CSF flow routes have been appreciated for nearly a century since Cushing proposed the 'third circulation'. Notwithstanding a substantial body of evidence, some recent fluid modeling has de-emphasized the primary pathway of CSF percolation from the ventricles to the basal cisterns (Oreskovic and Klarica, 2014a).Therefore, it is worth reemphasizing (Table 2) that, unlike the Mississippi River that flows unidirectionally "downward", the CSF has gentle but bidirectional ebbs and flows that create mixing.Overall, the *net* flow in adults is through the ventricles to the basal subarachnoid spaces (Bateman and Brown, 2012; Dreha-Kulaczewski et al., 2015). [See Hladky and Barrand (2014) for a detailed discussion of bulk flow, convection and diffusion.] Neurosurgeons understand well these anatomic and physiologic factorsbecause of the severe consequences of obstruction to the ventricular flow or overproduction of CSF (e.g., CP hyperplasia or papilloma) that cause hydrocephalus (Tisell, 2005; Warf et al., 2012; Bateman and Siddique, 2011; Spector et al., 2015). To ameliorate hydrocephalusin some cases, neurosurgeons either remove both lateral CPs to decrease CSF production (by presumably ~50%), or perform shunting of CSF to the peritoneal cavity(1).

Immunologic and biochemical homeostasis of CSF: relationship to ECSB

Since Cushing's work (1925), investigators have increasingly realized the complexity of the CSF, its functional relationship to the ECSB, and the hundreds of CSF functions that rely on transport, synthetic, metabolic, and transformative processes in CP or brain (Davson and Segal, 1996; Ghersi-Egea et al., 1994; Spector et al., 2015; Spector and Johanson, 2014).

A major difference between the CNS and the rest of the body is normally there are very few immunoglobulins in CSF and negligible white cells, unlike the plasma and lymph. Instead,

the immunologic protection of the CNS is due to the turnover of CSF in close contact with the ECSB and the extensive hyperactive microglial system in brain (Wake et. al, 2009; Verkhratsky and Butt, 2013). Microglia are evenly dispersed throughout the brain, have multiple protective and sculpturing functions, (e.g., removing unwanted, damaged or unused neuronal boutons) and are "probably the most active cells in the brain, constantly extending and contracting their processes to survey their environment" (Verkhratsky and Butt, 2013). As a consequence, there is continual cleansing of the ECSB. This is important for CSF physiology because of the ready interchange between the 'third circulation' CSF and the ECSB.

It is worth noting thehypothesis that in the elderly there is either senescence and/or immune suppression of the brain's microglial system.As a consequence, there is neuronal cell loss and build-up of dysfunctional proteins, peptides and substances like lipofuchsin, with adverse functional consequences e.g., Alzheimer's disease (Streit and Xue, 2010; Kan et al., 2015). In other words, the CSF composition depends not only on CP secretory processes but also the metabolic/transport phenomenain neurons, microglia and astrocytes. Hencethe appearance of leukocytes in CSF is a sign of neural damage and/or inflammation and, as noted above, important to evaluate after experimentallymanipulating the CNS.

A remarkable aspectof CSF is the complexity and function of the homeostatic systems for many substances in CSF, e.g. K^+, that depend on activity by both CP and astrocytes. Two more examples of hundreds are the relative stability of CSF concentrations of nucleosides (Spector and Johanson, 2007) and certain vitamins (e.g., vitamin C, folate, thiamine and pyridoxal monophosphate) even when there are large fluctuations in plasma concentrations (Spector, 2014). Each one of thismyriadof transport/metabolism/regulatory systems is typically separate, biochemically complex and exists in the CNS of most mammals from rodents to humans

(Davson and Segal, 1996; Miller, 2004; Spector, 2014; Spector et al., 2015). A monograph could be written about each system. Lack or malfunctioning of some of these systems is incompatible with life, e.g., the SVCT-2 in CP and neurons;or causes severe disease, e.g., the lack of the folate transport system in CP (Spector, 2014).

CSF as a "nourishing liquor"

Nourishment,used literally, has two definitions: first, food or other substances necessary for growth and health of the body and mind. Secondly, a narrower definition applies only to organic molecules. CSF secreted by CP contains in a controlled fashion, inorganic ions (Na^+, Cl^-, HCO_3^-, Ca^{++}, Mg^{++}, Mn^{++}, and PO_4^{---}) as well as certain vitamins, glucose and other organic molecules (Table 1).

Although most macronutrients (e.g., glucose, amino acids, lactate, fatty acids) and certain micronutrients (e.g., riboflavin, biotin, pantothenic acid and thiamine) enter brain mainly from blood directly through the BBB (Davson et al., 1987; Spector, 2009: 2014), a few substrates, e.g., vitamin C and methyltetrahydrofolate [folate], are transported solely or mainly from blood via the CP into CSF and then diffuse slowly into ECSBfor delivery to brain cells (Spector and Johanson, 2007; 2014: Spector, 2014). This CP-CSF-ECSB route applies to several micronutrients (e.g., ascorbate and folate),certain ions (e.g., Na^+, $Cl^-$and HCO_3^-), and perhaps some trace elements, e.g. Mn^{++},that when infused intravenously over ~1 h (as 0.9 mmole/kg $MnCl_2$) displayed initial uptake by CP, then CSF and finally brain regions(Aoki et al., 2004).This CP-CSF-ECSB distributional nexus must now be considered as one of the major conduits of supply for brain (Spector et al., 2015).Knock-out in animals (or in humans) of some of these systems in CP is neurologically devastating or fatal. On a normal human diet, for example,folate

or vitamin C is unable to diffuse extensively enough through the BBB if the folate or vitamin C systems in CP are knocked out (Spector, 2014; Spector and Johanson, 2014).

Moreover, several of these CP ionic and micronutrient transport systems, notwithstanding statements to the contrary, remain active and efficient into old age (Spector and Johanson, 2013). In elderly and Alzheimer's patients, the ionic make-up of the CSF, the concentrations of CSF vitamin C and folate (2-4 x higher than plasma), and the CP synthesis of transthyretin and transfer into CSF are maintained (Spector and Johanson, 2013). In fact, there is more vitamin C in brain in normal elderly than younger humans by MRI spectroscopy (Spector and Johanson, 2013). Thus, the notion of the CSF as nourishing liquor for brain has validity for many ions and a few organic molecules, but not for macronutrients.

CP either transports peptides and proteins from blood into CSF (e.g., leptin),or synthesizes others and releases them into CSF (e.g., transthyretin, BDNF, and IGF-2). These peptides and proteins in CSF can then readily diffuse into brain (Johanson et al., 2011a; 2011b). Exact roles of these proteins in adults are not entirely clear but as discussed below, their diminishing synthesis in rodents coincides with brain "aging". This phenomenon may occur in humans; in fact, there is already preliminary evidence, a possibility that needs intense investigation (Baruch et al., 2014). At present, the best hypotheses for the presence of these peptides and proteins (e.g., IGF-1 and BDNF) in adult human CSF (and ECSB) is to facilitate the low level replication of neurons in the human dentate gyrus and striatum, and to maintain and preserve neuronal function and longevity (Baruch et al.,2014). Some CSF peptides (e.g., leptin and IGF-1) may also be transporting signals for on-and-off functions of target neurons in brain (Rodriguez et al., 2010; Johanson et al., 2011a).

Extra-choroid plexus formation of CSF

For decades, there has been interest inan extra-CP source of CSF (Hammock and Milhorat, 1976).One groupeven claims that *most*CSF comes from cerebral capillaries into ECSB, with subsequentconvection/diffusion into the ventricles/subarachnoid space (Oreskovic and Klarica, 2010; 2014a;b). A review of voluminous data, however, concludedthat in rodents extra-choroidal CSF production was <10% of total fluid formation (Davson et al. 1987; Davson and Segal, 1996). Particularly revealing has been the finding that in un-instrumented but anesthetized rats, tracer concentrations of Na^+ and Cl^-, the principal components of CSF, readily passed by active transport through CP into CSF and then trans-ependymallyinto surrounding brain (but not across cortical capillaries) at early time points (Smith and Rapoport, 1986). A low-level diffusional exchange of $Na^+$from blood through cerebral capillaries into ECSB (and back) could not be ruled out.The permeability constant (P) for $^{22}Na^+$ into cerebral cortex was akin to passively-distributed,H_2O-soluble mannitol;and $P^{36}Cl^-$ was only 1/2 that of mannitol, a much larger molecule (MW =180).This indicatedthat Cl^- passage through BBB is by simple diffusion. Later, employing prolonged (10 min) in situ perfusion in heavy-instrumented barbiturate-anesthetized rats, Ennis et al. (1996) confirmed that Na^+ did not pass through the BBB more rapidly than their larger passive marker,α-aminoisobutyric acid (MW = 103); however, they detected a small (~20%) saturable component when the Na^+ concentration was reduced from 138 (normal) to 0.2 mM. Whether such a low concentration of Na^+ would change the permeability characteristics for Na^+ at the BBB is unknown, and whether this study actually measures transfer through the capillaries or just exchange with Na^+ in the capillaries, especially at 0.2 mM, are also unknown. These later prolonged experiments of Ennis et al. (1996) must be viewed in the context of the disadvantages associated withheavily-instrumented, barbiturate-anesthetized rats.

Recently, Mokgokong et al. (2014) stated that for cerebral capillaries to secrete Na^+, Cl^-, K^+, HCO_3^- into the ECSBwith subsequent flowinto CSF, there must be multiple specific ionic transporters at both the luminal and abluminal sides of cerebral capillaries for *net*unidirectional ion transport through BBB. To their credit, these authors made heroic efforts to find in cerebral capillary endotheliumthosefluid-translocating transportersexisting in CP and kidney. Using histological techniques that easily detect ion transporters in CP and kidney, they found no evidence for such transporters in cerebral microvessels. Mokgokong et al. (2014) then employed ultrasensitive immunohistologybut found only low-level evidence for just several ion transporters (AE2, NBCe1 and NBCn1) in vivo, but with poor localization. In *cultured*cerebral endothelial cells (rats), several other relevant ion transporters were identified (Mokgokong et al., 2014). However, cerebral cortical endothelial cells are well known to de-differentiate in culture. Therefore, with these meticulous studies the authors found no evidence for Cl^- or K^+ transport across cerebral capillaries, even in culture. Overall this recent study fitsearlier conclusions (Davson and Segal, 1996; Spector et al.,2015) ofnegligible evidence for extra-choroidal secretion of fluid via the ECSB into CSF,since$Na^+$and $Cl^-$are not significantly secreted through cerebral capillaries.

Notwithstanding the above evidence, several authors persistently deny that CP is the sourceofCSF,but rather maintain thatCSF originates from cerebral capillaries (Oreskovic and Clarica, 2010; 2014a; b). Their data and interpretations have been debunked by Hladky and Barrand (2014), Bateman and Brown (2012), Brinkman et al. (2014) and Spector et al. (2015).Wereiteratethat a principal error is notdistinguishingH_2O *exchange* fromCSF formation (Davson et al., 1987; Hladky and Barrand, 2014; Spector et al. 2015). Water in humans and animals rapidly exchanges among blood, brain, and CSF. Water exchange is >100 x the rate of

formation of CSF – a fluid consisting mainly of Na^+, Cl^-, and HCO_3^-. In fact, in one circulatory pass through rat CNS, >50% of H_2O in blood *exchanges* equally with H_2O in brain (Bateman and Brown, 2012; Spector et al., 2015). Factors other thanH_2O channel transfer may be operativesince aquaporins have not been identifiedin BBB endothelium or at the basal side of adult CP.

The choroid plexus/CSF axis analogy to glomerular filtrate/kidney

J.R. Pappenheimer et al. (1961, 1962) demonstrated in conscious goats that CP-CSF,in removing organic acids, from CSF behaveslike kidney in removing such compounds from blood by saturable transporters(Spector and Johanson, 1989). This analogy has substantial merit. Human glomerular filtrate (~200 l/d of clear fluid containing salts, vitamins, peptides, and amino acids as well as unwanted waste catabolites and foreign molecules) is differentially handled. Water, salts, vitamins and peptides are mainly *reabsorbed* whereas the waste productsleave in the urine. Moreover,specific renal abluminal transporters “pump” (secrete) unwanted molecules from blood into urine. On average, each gram of human kidney reabsorbs ~0.3 l/d of fluid.

A similar physiology occurs in CP by comparable, sometimes identical, mechanisms. CP secretes ~0.2 l of CSF/d/g of CP (Table 1) – a process similar to the carrier-mediated reabsorption of the ‘requisite’ molecules in glomerular filtrate (into blood) by the renal tubules. However, there are major differences. Renal reabsorption of H_2O from glomerular filtrate, for example,depends on aquaporins and anti-diuretic hormone whereas CP secretion is not or to a much lesser degree dependent on AQP-1 on the apical (CSF) side of CP (Spector et al., 2015).

On the other hand, there are marked similarities between choroidal and renal systems. AA is pumped into CSF by SVCT-2 and in kidney reabsorbed by SVCT-1, a closely-related transporter in structure and function. In kidney when the maximum ability of the tubules to

reabsorb AA is exceeded, the excess is excreted in the urine. This saturating phenomenon provides an important part of the homeostatic system for AA in the body (Spector and Johanson, 2006). Recently identifiedare many polymorphisms of SVCT-1 that, although all are functional, displayvariations possibly explaining the differential sensitivity of humans to scurvy (Timpson et al., 2010; Spector and Johanson, 2014). In other words, some individuals might reabsorb AA more efficiently from glomerular filtrateand thus be less dependent on dietary AA. Awaiting determination is whether (although likely) there are similar polymorphisms of human SVCT-2 in CP and neurons, with functional consequences. As noted above, AA exemplifiesa substance that enters the ECSB and then brain cells predominantly by the blood-CP-CSF-brain route via SVCT-2 at the BCSFB. SVCT-2 is also responsible for AA uptake by neurons containing ~10 mM AA compared with ~0.05 mM in plasma. The AA-concentrating system in central neurons is powerful indeed (Spector and Johanson, 2014), relating to anti-oxidant,cofactor as well as DNA metabolic roles.

A more complex system in both kidney and CP/brainis the handling of riboflavin. In human kidney, riboflavin is reabsorbed by the tubules with a half-saturation concentration (K_T) of ~0.2 μM (Spector, 2014). When the plasma concentration rises above ~0.5 μM, the renal tubular transporter is saturated and excess riboflavinis excreted in urine. Moreover, riboflavin also has affinity for OAT-3, a promiscuous transporter that pumps penicillin and cimetidine into urine and also, with a $K_T$of ~70 μM for riboflavin, pumps the latter into urine (Spector and Johanson, 2006; Spector, 2014). Thus, the kidney has a dual system (i.e., *reabsorption* at the normal plasma concentrations and *secretion* at higher blood concentrations) for regulating the excretion and thus the blood concentration of riboflavin.

In the CNS, in many casesthe transport/regulatory systems are extremely effective since the brain generally requires stable metabolite and ion concentrations to function effectively (Davson et al., 1987; Spector, 2009; Spector and Johanson, 2006; 2007; 2014). For example, in the CNS, riboflavin entry into and exit from brain are complex but somewhat different than in kidney. Riboflavin is transported into brain by a recently-cloned transporter at the BBB with a K_T of ~0.2 μM (Spector, 2014). Riboflavinis also transported out of brain and CSF by the promiscuous higher affinity OAT-3 located at both the abluminal side of brain capillaries and the apical side of CP (Spector, 2014). Intraventricularly-injected riboflavin in conscious rabbits is cleared more rapidlyfrom CSF by OAT-3 (and possibly other transporters) than penicillin. Clinically there are patients without the riboflavin transport system at the BBB that suffer a severe neurological disorder,due to the crucial roles that this vitamin plays in brain function (Spector, 2014). Thus, like kidney, the CNS has dual regulatory transport systems for riboflavin at both the BBB and CP.

A similar situation exists for plasma nucleoside (e.g., uridine) and copper transport into brain with saturable *influx*at the *BBB*, and separate clearance *(efflux)*mechanisms for nucleosides and copper from CSF at the *BCSFB*(Spector and Johanson 2007; Fu et al., 2014). These examples represent aplethora (hundreds) of similar functions between CP and kidney. There are many reasons, then, to call CP the‘kidney’ of the brain (Spector and Johanson, 1989).

Trans-ependymal and trans-pial exchange of materials between CSF and ECSB

In documenting CSF-ECSB exchange of molecules in adults, three critical points deserve emphasis. *First*, as noted above, H_2O is freely permeable among blood, brain and CSF. Thus, looking at H_2O exchange is not relevant to CSF formation, circulation or exchange with the ECSB for the purpose of this discussion, since the H_2O content of brain under normal

circumstances is constant. *Second*, since the originally-documented free exchange of dyes between CSF and ECSB, over 100 years ago (Saunders et al., 2014), the histologic explanation is now clear for this ready exchange of physiologic ions, small H_2O-soluble molecules (100-300 D) and the slower (passive) diffusion of even larger non-transported H_2O-soluble molecules (e.g., inulin; ~5 kD) from CSF into ECSB, or ECSB into CSF: in adults there are large*gap junctions* in the pia and ependymawhose permeable molecular structure provides the route for CSF–ECSB diffusion and exchange (Whish et al., 2015). The kinetics ofCSF-ECSB exchange of H_2O-soluble molecules, not transported by carriers,is a complex mixed process depending on the principles of diffusion (e.g., molecular size, H_2O-solubility and ionic nature) and tissue microarchitectureof the cell-interstitium relationships(e.g., tortuosity and viscosity of the ECSB matrix). Hydrodynamic factors affecting molecular movement throughout ECSB relate to heartbeat, respirations, posture and time of day.Passive molecular exchange and diffusion (but not CNS H_2Ofluxes), it must be emphasized, is a slow process. In CSF-ECSB molecular exchanges, the experimental tracer equilibration with deepest parts of the brain from CSF (~1.5 cm in humans) takes many hours or even days. (See Hladky and Barrand, 2014 and references therein for a thorough discussion.) *And third,* for best evaluation of CSF-ECSB molecular exchanges,it is imperative to carry out nearly-ideal replicated experiments to avoid misleading results due to the potential technical problems described in Table 3.

To focus on near-ideal instructive experiments, Hammarstrom (1966) injected intravenously ^{14}C-ascorbic acid (AA) (the reduced form of vitamin Cfound in plasma; Spector and Johanson, 2006) into *conscious* mice and performed autoradiography at subsequent time points. He showed that ^{14}C-AA first entered CP, then CSF, next the brain closest to the CSF and finally the whole brain. Hammarstrom's observations were extended in several series of studies

in *conscious* rabbits with either intravenous or intraventricular injection of ^{14}C-AA, (Spector,1981; Spector and Lorenzo, 1973; 1974; Spector et al.,1977). Intraventricular injections (0.1 ml) in these studies were done under sodium pentothal anesthesia; the rabbits awoke quickly thereafter. Also the number of leukocytes in the clear cisternal CSF 2 h after the injection was quantified and averaged at<50 cells/mm^3, thus showing minimal inflammation. For humans, there is compelling evidenceboth direct and indirect, that the AA transport systems in CP and brain resemble those in mice and rabbits (Spector and Johanson, 2006; 2013). We also confirmed that the passively-distributing mannitol, sucrose and inulin in intraventricularly-injected conscious rabbits readily diffusedacross the ependymal and pial interfaces into brain as a function of molecular size and time (2 vs. 4 h) with mannitol > sucrose > inulin, as expectedfrom the molecular size gradation (Spector and Lorenzo, 1974; Spector, 1981).

In an elegant autoradiographic study in conscious rats into which a catheter had been implanted 3 d before, Proescholdt et al. (2000) demonstrated how inulin diffuses into rat brain after lateral intraventricular CSF injections. They elegantly confirmed an initial substantial diffusion of the test markerthrough the ependyma, then gradually over time deeper into the para-ependymal brain and, as the CSF flowed into the subarachnoid space, through the pia into the surrounding brain. Although diffusion throughoutthe CNS was heterogeneous due to pooling in subarachnoid spaces and the heterogeneous anatomy of the brain, by 4 h the entire brain in these conscious rats was labeled with radioinulin. With the larger brains of rabbits (10 g) and humans (>1 kg), the process would take longer following ventricular administration (CSF as a source). As noted above, when tracer $^{22}Na^+$ or $^{36}Cl^-$ was injected intravenously into un-instrumented but anesthetized rats, there was a definitiverapid flux of these ions through CP into ventricular CSF, and thereafter through ependymal gap junctions into para-ventricular brain (Smith and Rapoport,

1986). Because the CSF and ECSB concentrations of Na and Cl are similar (~138 mM) and stable, these "fluxesare in reality exchanges between ventricular CSF and ECSB.Noteworthy (as discussed above) is that radiolabeled Na^+ and Cl^-in plasma donot pass readily through the cerebral capillariesexcept by minimal diffusion; this is consistent withno known transporter mechanism for NaCl to penetrate the BBB. Altogether the data support substantial transfer ofNaCl-rich fluid inwardly across the BCSFB and then thepermeable CSF-brain interface, but *not acrossthe BBB*into the CNS.

On the other hand, there is ready diffusion of *waste products*fromneural/glial metabolism, vectorially out of the brain from *ECSB into CSF*. Two examples are homovanillic acid (HVA) from dopamine metabolism and 5-hydroxyindoleacetic acid (5-HIAA) from serotonin metabolism. In both animals and humans, these metabolites have high concentrations in brain and lateral ventricles, less in the 3rd and 4th ventricles, and a very low level in the lumbar sac (Davson et al., 1987). This is because the CP has efficient transport systems to remove HVA and 5-HIAA derived from brain (Barkai et al., 1972). The aforementioned example clearlydocuments a bidirectional flow of molecules between the ECSB and CSF, in this case into CSF. Thus, there is *not just* bulk flow of CSF through the ventricles, cranial subarachnoid and spinal spaces back into blood, but there are also passive molecular movements into, and from, the CSF and ECSB. Moreover, molecules like HVA and 5-HIAA distribute not only by passive diffusion/convectionwithin the CNS but also are *actively* pumped out ofECSB by BBB transporters,and out of CSF by CP transporters.

For an individual drug molecule, its penetrating characteristics into brain after intraventricular injection (or entry from blood into CSF) will depend on lipid solubility, charge, size and ability to be transported on carriers, or not. Take penicillin and methotrexate, for

example,that rapidly disappear after intraventricular injection by bulk flow and carrier-mediated transport by OAT-3 in brain capillaries and CP (Spector, 2010; Li et al., 2013). Accordingly, thesedrugs in transit are actively-reabsorbed and thus appear to penetrate less deeply into brain from CSF, unlike molecules like mannitol that are not actively removed but exit CSF simply by diffusion (minimal) and bulk flow.

The statement that molecules in ventricular CSF "minimally enter the brain parenchyma" via the ependyma reported by Iliff et al. (2012) seems doubtful in many cases –certainly for passively-distributedH_2O-soluble molecules and those that do not pass though the BBB and BCSFB except minimally by simple diffusion. This statement by (Iliff et al., 2012) is based on*short-term* 30-min studies in heavily instrumented anesthetized mice employing fluorescent markers (of unknown characteristics) with molecular weights of ~1, 3, and 2000 kD. The longer-duration CSF studies in conscious animalsof extensively-permeatingnon-electrolytes (with non-carrier transported mannitol, sucrose and inulin) belie the conclusion by Iliff et al. (2012) of negligible transependymal exchange.

In conceptualizing the CSF-ECSB relationship, one can visualize the CSF-ECSB as, in a sense, one large compartment (~300 ml of CSF + ISF in humans) with the ECSB having many tortuous arms, like the Buddhist Kwan Yin with a'thousand arms'. The human ECSB makes up ~15% of brain volume,situated behind the BBB.When thinking about molecular fluxes throughout this extracellular compartment, there are *four*situations to consider. *First*, with substances like Na^+, Cl^-or AA that donot pass through the BBB appreciably, or are not metabolized or significantly degraded, and are pumped into CSF in a steady fashion so that the CSF concentration of these molecules is ~ stable, there is slow movement (mainly diffusion) over time (hours to days) from CSF into even the deepest recesses of the ECSB; the integrated

resultis approximate equalization of concentration throughout the entire CSF-ECSB compartment. Moreover, at steady state there is continual exchange and mixing within the compartment but no *net*flux between ECSB and CSF. A predictive model of this situation was presented for AA (Spector et al., 1977). This model, which assumed at steady state that the CSF-ECSB was one compartment for AA, showed that the K_T (the half saturation concentration for AA transport by CP) was optimized to minimize CSF AA fluctuations in the face of varying blood concentrations of AA. Moreover, this model correctly predicted CSF and brain AA concentrations when tested in vivo in rabbits (Spector et al., 1977). Thus the AA system in CP and neurons, in series functionally, provided excellent homeostasis in brain for AA.This is the reason that the brain is the last organ to become depleted in dietary AA deficiency (Spector et al., 1977). Many other transportersat the BBB and/or CP also act as regulatory systems in that their one-half saturation concentration is close to the blood level; thus if the blood concentration is raised*relatively* less of the molecule is transported into CNS and vice versa (Davson et al., 1987;Spector et al., 1977).

A *second* situation described by Barkai et al. (1972) is when the brain *synthesizes*substances not present in nascent CSF, e.g., HVA and 5-HIAA, that diffuse from the ECSB into CSF, and are then carried out of CSF by active transport at the CP andbulk flow into venous blood/ lymph. This is the so-called *sink* effect (i.e., sweeping or cleaning action) that lowers the concentration of substances, endogenous ions as well asnon-electrolytes (Parandoosh et al., 1982), in CSF to protect the brain environment (Davson et al., 1987).

Third, in some cases the CSF serves as a *donor*or sourcefor thebrain. This pertains to molecules in CSF either manufactured by CP like transthyretin, IGF-2 and BDNF, or pumped into CSF from plasma, e.g., IGF-1, prolactinand leptin. These moleculesthen allgradually move

(from ventricles or subarachnoid space) into the ECSB to carry out functions in glia, neurons and endothelium.

Fourth, and finally, in considering molecular fluxes within the CSF-ECSB compartment, many H_2O-soluble molecules including drugs and toxinsinjected into blood are unable to pass through the barriers, or if they can penetrate into CNS,donot accumulate to therapeutic (e.g., penicillin) or toxic (copper) levels.In many cases, they are pumped out of ECSBby extrusion systems incerebral capillary endothelia (e.g., by P-glycoprotein),and out of CSF by CP efflux transporters (e.g., by OAT3);the compromise of these excretory systems may lead to central buildup of uremic toxins (Hosoyaand Tachikawa, 2011).Xenobiotics are alsoenzymatically degraded by endothelial or epithelial cellswithin these barriers (Ghersi-Egea et al., 1994). Still, many beneficial endogenous solutes distribute bidirectionallywithin the CSF-ECSBcompartment, and have a 'protected sanctuary'(free of toxins) in which ECSB homeostasis occurs so that brain functions properly. On the other hand, penicillin exemplifies a 'foreign' but therapeutically-important drug largely excluded from the CSF-ECSB unless very large amounts of the antibiotic are administered.

BCSFB and BBB drug transfer into and out of CSF and ECSB

Drug transfer into and out of the CSF (and ECSB) depends on molecular size, lipid solubility, ionic charge and protein binding; and also, on affinity for influx or efflux carriers at the BCSFB and BBB (Davson et al. 1987; Spector 2010). There are three types of carriers: facilitated diffusion, primary or secondary active transporters, and receptor-mediated transporters (often through vesicles).

Two examples of important molecular transport of foreign molecules into CSF through the BCSFB are iso-ascorbic acid (IsoA) and scyllitol.IsoA is a widely-used antioxidant which

travels on the AA system in CP into CSF, and thereafter diffuses into the ECSB (Spector and Lorenzo, 1974). IsoA has ~20% the affinity of AA for the SCVT-2 transporter at the CP and neurons. Thus ~20 xthe dose of IsoA vs. AA is able to substitute as an anti-scorbutic agent in brain since IsoA is just as effective an enzyme co-factor, e.g., for dopamine hydroxylase, as AA (Spector and Lorenzo, 1974). Secondly, the natural product scyllitol (an isomer of myo-inositol) is now being tried as a CNS drug. Scyllitol has the same affinity as myo-inositol at the CP and BBB for transfer *into* CSF andthe ECSB (Spector, 1978).

Other water-solubleionic drugs also readily enter the brain via saturable carrier-mediated systems at the BBB. An example is the antihistamine diphenhydramine (DPH),widely employed as a sleep aid (Goldberg et al., 1987).Unlike molecules like diazepam for anxiety and thiopental for anesthesia,which are nearly completely cleared from blood into the ECSB in one pass of the circulation because they are highly lipid-soluble,DPH is actively transported at the BBB (by a molecularly uncharacterized transport system) and achieves higher concentrations in the ECSB and CSF than in plasma notwithstanding substantial protein binding in the latter (Goldberg et al., 1987; Sadiq et al., 2011). However, like riboflavin, DPH is rapidly cleared from CSF by a separate system in CP (Goldberg et al., 1987).

To place these transport systems in pharmacologic perspective, one needs to consider what happens to transported molecules that traverse the BBB and BCSF barriers by processes other than simple diffusion (e.g., carrier-mediated or endosomal-mediated). There are of course two main barriers, and molecules can potentially be transported in either direction. Glucose is transported from plasma through the BBB and BCSFB by the GLUT-1 transporter in the cerebral capillaries and CP basolateral membrane(Farrell et al. 1992). This is an example of “both into

CNS." Penicillin, on the other hand,typifies a molecule that both cerebral capillaries and CP transport out of ECSB and CSF, respectively, into blood–"both out of CNS".

Bidirectional transport, a significant feature of CNS barrier systems, has a bearing on pharmacokinetics and vectorial distribution. Riboflavin, nucleosides, copper and diphenhydramine (above) are molecules transported *into* the ECSB by different specific systems in cerebral capillaries, but are also transported *out of* the CSF into blood by different systems in CP. Moreover,molecules like AA can be transported into CSF from blood via CP and thence diffuse into the ECSB but, in its normal reduced form, is unable to broach the BBB for brain-inward transport. Finally, P-glycoprotein in cerebral capillaries but not CP is highly active in extruding (and thus excluding) a multitude of molecules from the ECSB. Thus, the routes into and out of brain and CSF tracked by many molecules (that do not traverse the BBB and BCSFB by simple diffusion)are, in many cases, complex.Multiple permeability, transport, vectorial and hydrodynamic factors, then, determine drug concentration in CSF and ECSB.

Bidirectional brain – choroid plexus molecular information flow via CSF

A recent fascinating report suggests that aging brain puts out 'signals' into CSF that interfere with CP production of neurotrophic molecules. Specifically, Baruch et al. (2014) demonstrated that aged mouse brains switch from healthy interferon (IFN-II) signaling to IFN-I signaling. By way of CSF distribution, IFN-1 caused the CP to slow down the production and secretion into CSF of factors such as IGF-1 and BDNF, molecules that "support neuronal growth, differentiation and survival" (Baruch et al., 2014). Presumably as a consequence, there was a decline in cognitive function and hippocampal (dentate) neurogenesis. This decline was prevented by reestablishment of IFN-II-dependent CP activity that, in mice, had been lost in aging (Baruch et al., 2014). Whether this occurs in human CP is yet to be established but, if so,

opens wide and actionable possibilities. In aged humans, Baruch et al. (2014) corroborated a marked increase in IFN-II in CP at post-mortem examination. At least in mice, this specific aging process is preventable and shows once again the critical role played by the CP-CSF axis not only in maintaining adult brain, but also in essentially preventing neural decline in aging. This introduces the concept of bidirectional signal (information) flowin the brain-CSF-CP axis.

Ventricular transmission of cells andmolecularsignalstransported across ependyma and choroid plexus into CSF

The continual, directed flow of CSF is essential for: i) delivering to targeted CNS regions certain cells/factors transported into the ventricles, and ii) maintaining the physical configuration of the ventricular system to assure proper CSF volume and dynamics. Although bulk flow through the core of the ventricles is rhythmically-driven by cardio-respiratory pulsations (Bottan et al., 2012), the finer movement of CSF along the ventricular walls is energized by wave-like motion of motile cilia projecting outward across the apical membrane of ependymal cells. At least in rats, ependymal ciliary activity helps provide a 'guided flow' (streaming) of nascent neurons (derived from subventricular neurogenic zones) rostrally to olfactory regions(Sawamoto et al., 2006). Unlike in rodents, however, in adult humans there is evidently no birthing of neurons in the subventricular zone destined for transport down the Rostral Medial Stream for olfactory renewal; thus, the need for such a ciliary-dependent mechanism to modulate adult neurogenesis has not been demonstrated in humans.

Normal ependymal ciliary and gap junctional functions are needed to provide the requisite development of ventricular shape and size, and normal 3rd ventricle-aqueductal CSF flow to the 4th ventricle. Rare ciliary deficiencies in the axoneme motor protein dynein (Bryan et al., 1983), collectively known as primary ciliary dyskinesia (PCD), cause hydrocephalus by attenuating

cilia-promoted CSF streaming along ependyma; this hydrocephalus, confirmed by sonographic marking (Wessels et al., 2003), is associated with aqueductal stenosis (Vieira et al., 2012)). The insertionally-mutated axonemal dynein heavy chain gene in mice (Mdnah5) also occurs in humans (DNAH5) with PCD (Ibanez-Tallon et al., 2002). PCD-associated abnormal ciliary motion is either absent, hypoactive or reversed. PCD is the hallmark ofmany mouse ependymal ciliary defect models (Lee, 2013), but this Kartagener syndrome with hydrocephalus is rare in humans, i.e. ~1/30,000 (Berlucchi et al., 2012; Bush, 2000; al-Shroof et al., 2001). The *hpy* mouse model of post-natal hydrocephalus, like human PCD, has an ependymal dynein-deficiency frequency of ~35%; and advantageously (experimentally) lacks the respiratory ciliary problems (moving mucus) that attend Kartagener syndrome. Murine models of PCD, by revealing strain-specific differences in phenotype, inform on genetic mechanisms that modify susceptibility to hydrocephalus linked to ciliary dysfunction (Lee, 2013). On the other hand, ependymal *gap junctional* defects of the cadherin gene disrupt the 3rd ventriculo-aqueductal configuration and functions, leading to congenital hydrocephalus in rodents as well as humans (Rodriguez et al., 2012; Guerra et al. 2015). Clearly, the molecular integrity of the ependymal proteins, dynein and cadherin, is essential to maintain normal ventriculo-aqueductal flow and support certain brain functions undergirded by supply (bulk flow delivery) of CSF neurotrophins.

Key neural functions modulated by CSF-borne peptides and proteins include neuronal plasticity, immunomodulation and neurogenesis. Neurotrophins and growth factors such as IGF-1, BDNF and VEGF are secreted by CP into CSF, and then while movingthrough the ventricles, are taken up across the permeable ependymal interface into various regions (e.g., hippocampus, caudate nucleus, hypothalamus, periventricular white matter and periaqueductal gray). Upregulation of the choroidal content of peptides such as IGF-1, BDNF and VEGF, and their

subsequent release to CSF for distribution to neuronal targets, are likely part of an integrated feedback system (sensory and effector components) involving biochemical information processing by T cells in both brain and CP (Schwartz and Baruch, 2012). Thus, choroid epithelial cells likely act as mediators of peptide signal transfer in an immunomodulatory process that ultimately buffers the brain behavior against episodes of systemic (peripheral) stress. Moreover, as demonstrated in adult animals, the CSF is an important medium for supplying factors to regulate neurogenesis in the dentate gyrus and some subventricular zones (Johanson et al., 2011a). Perhaps in humans, IGF-1 and BDNF flow from the CP-CSF nexus to stimulate nascent neuron production in the sub-ventricular zone for the striatum (Ernst et al., 2014). However, additional evidence is needed to bolster the notion that the sub-ventricular zone in adult humans isa significant generator of new neurons for the brain.

The 'glymphatic' system: Is there a quasi-lymphatic drainage of CSF and ISF?

Recently, notwithstanding the overwhelming evidence for bidirectional flow of molecules between the CSF and ECSB via gap junction pores in the ependyma and pia, the group led by Nedergaard has made three startling claims based in large part on fluorescent studies in anesthetized, heavily-instrumented mice and rats (Iliff et al. 2012; 2013; Xie et al., 2013). *First*, these authors make the incorrect claim of minimal exchange between CSF and brain through the ependyma, which ignores the evidence presented above. Proescholdt et al. (2000) provided a convincing autoradiographic demonstration of intraventricularly-injected ^{14}C-inulin flowing from the lateral ventricle of conscious rats into periventricular brain,as a function of time;they observed diffusion throughout the entire brain at 4 hr. *Second*, the Nedergaard group (Iliff et al., 2012; 2013) claims that CSF flows mainly down the sheaths of arteries from the pial side, then into the ECSB,finally returning back to the subarachnoid space via sheaths of veins carrying

small molecules and peptides out of brain into CSF for exit into blood by bulk flow. They name this hypothesized flow route the "glymphatic" pathway analogous to the lymph system in the rest of the body. *Third*, they also claim (Xie et al., 2013) that in 15 min after the onset of sleep or anesthesia, the ECS of mouse brain expands from ~14% to 23%,i.e., a ~60% increase based on observations of fluorescent marker penetration up to 0.2 mm into mouse cortex. They generalize this observation to the entire brain,and suggest that one function of sleep is to clear out the waste products of metabolism through the expansion and increased flow of CSF throughout brain during sleep.

In response to their second claim about the existence of the "glymphatic" system, Hladky and Barrand (2014) and Brinker et al. (2014) make a convincing case that these arguments are unlikely to be correct and may not apply to humans. To summarize their objections and our own critique of the hypothesized "glymphatic" circulation, it should be noted that the lymphatic circulation (content, flow and anatomy) is nothing like the CSF composition, flow and "anatomy." *First*, the milky lymph contains 100 x more protein than CSF; moreover,lymph formation is due to the *passive* non-specific outflow of protein and smaller molecules out of the vascular circulation into tissue (e.g. muscle)ECS (Starling mechanism) (Hladky and Barrand, 2014). From the ECS, the lymph flows into lymphatic vessels that depend on muscle contraction, gravity and pressure factors to return lymph to venous blood. In contrast, the CSF is an *active* secretion of CP that has a myriad of functions. *Second*, the authors do not acknowledgedecades of data on ependymal/pial permeability and exchange as noted above. *Third*, there is contrary data that molecular flow is along the arterial sheaths *into*subarachnoid CSF or is at least bidirectional in the peri-arterial sheaths. Recent data by Whish et al. (2015) suggest that the para-arterial CSF is stagnant. *Fourth*, others have not observed (with fluorescent markers) any flow along the

cerebral veins. *Fifth*, results in aquaporin-4 KO mice are not consistent with their hypothesized "glymphatic" pathway. *Sixth*, the mice in experiments by Iliff et al. (2012) were heavily instrumented and pressure injected;the condition of these mice at the end of the experiments was not reported. For further discussion, see Hladky and Barrand (2014) and Brinker et al. (2014). To recapitulate, the notion that there exists a "glymphatic" pathway for CSF exchange with ECSB remains at best, a working hypothesis and, in our view, unlikely to be more than a minor phenomenon of CSF dynamics. An analysis of the extant data, e.g., the ideal or near-ideal experiments in conscious animals (Hammarstrom, 1966; Spector, 1981; Proescholdt et al., 2000) suggest that molecular flux of molecules not transported by specialized transporters is due mainly to diffusion through the ependymal and pial gap junctions; and that this is the major route of exchange between CSF and ECSB (Whish et al., 2015).

The initial notion in the third part (above) of the proposed "glymphatic" hypothesis is that the brain doesnot have a sophisticated immunological (clearance) system. In fact, the brain does. The brain is normally cleansed by microglia that are evenly and diffusely present throughout the entire brain (Wake et al., 2009).These hyperactive cells are continuallyextending and retracting their processes, taking up debris (e.g., dead cells, damaged proteins and unused/injured synaptic boutons). Moreover, at the BBB and CP there are carriers for peptides($A\beta_{40}$) and small organic molecules thattransportunneeded molecules out of ECSB-CSF. At the ependyma and pia, molecules such as HVA and 5-HIAA diffuse out of ECSB into CSF for removal by carrier-mediated transport and bulk flow.In Alzheimer's disease, the concentration of $A\beta_{42}$ in CSF is on average lower than in normal age-matched controls. Active carrier-mediated transport processes that remove Aβ from CSF (Pascale et al., 2011) do not resemble the *passive* lymph circulation.

Thus, the notion of CSF being like the lymphatic system is not a good analogy and should be abandoned.

The second part of the third hypothesis (above) of the Nedergaard group, viz., that the ECSB during sleep and anesthesia increases by ~60% within 15 min (Xie et al., 2013), seems an inappropriate global extrapolation from studies of brains, in heavily-instrumented mice, to a depth of 0.2 mm. Coupled to this notion is the claim that sleep "drives metabolite clearance from brain." Again Hladky and Barrand (2014) and Brinker et al. (2014) argue against these conclusions.

Many physiologic parameterschange during sleep. For example, in humans, there is impressive diurnal variation in CSF production, more at night. What happens in mice? Are the adrenergic blockade experiments reported by Xie et al. (2013) really demonstrating that the adrenergic system is constricting the ECSB in conscious awake mice, or are they just increasing CP blood flow and CSF production? Second, impedance measurements during sleep are not consistent with a 60% increase in the ECS of brain. Most challenging to their hypothesis is the question: where does the increase in ECSB (with a composition similar to CSF) come from? Since cerebral capillaries are basically impermeable to NaCl (Smith and Rapoport, 1986), from what source would a 60% increase in human ECSB (~90 ml) originate in 15 min? It probably does notcome from CP and CSF production. The human CPs makes ~0.4 ml/min. Even if CP production of CSF doubles during sleep and all the extra CSF went into the ECSB, that volume would only be ~6 ml in 15 min. See Brinker et al. (2014) and Hladky and Barrand (2014) for detailed comments on these points. Finally, how about naps? Ten-minute naps during the daytime for many humans are refreshing. In that time frame, is there a huge expansion of the ECSB and a significant increase in metabolite clearance? To summarize the proposed

'glymphatic system', based on extant data and the collective considerations addressed above,it seems unwarranted to conclude that the entire brainECSB during sleep expands by 60% in 15 min, therebymetabolically cleansing the wholeCNS.

Viewpoint perspective on the cerebrospinal fluid

Rather than looking at CSF as part of a glymphatic or quasi-lymphatic system,perhaps a goodway to viewCSF is that of a multifunctional fluid in close contact and interacting with the ECSB. In many respects, the CSF-ECSB can be considered a single 'collective' compartment. In other words, holistically, the CSF and ECSB should not be considered alone but rather as a combined but sometimes inhomogeneous compartment. However, for certain molecules (e.g., K^+ and AA) that are continuously secreted by CP into the ventricles and leave CNS by routes noted above, there are sometimes large fluctuations in their concentrations in local regions of the ECSB due to, e.g., intense neuronal activity (Verkharatsky and Butt, 2013).In practice, however, suchinhomogeneities are quickly resolved by astrocyte buffering and diffusion of solute away from the highly active site. On the other hand, the concentration of some molecules, especially those destined for excretion out of CSF, are routinely quite inhomogeneous (e.g., HVA, 5-HIAA) in the CSF-ECSB compartment.Also to be considered are the myriad of other functions of the CSF, e.g., buoyancy, nourishment, and the transfer of growth and neuronal maintenance factors havingreceptor targets in various brain regions.

Conclusion

In this review, we portray the composition and numerous functions of CSF. Theseinclude an analysis of various analogical descriptions, the importance of CP in secreting CSF, and the ready CSF-ECSB molecular exchange. To do this, we focused on often-ignored methodological issues and used exemplary experiments to provide the best evidence for conclusions relevant to

human CSF. We also considerthe importance of the ECSB with its continuously-active microglial, neuronal and astrocytic cells affecting CSF composition. In actuality, the formation, flow and reentry of CSF into the blood was reasonably well described by Cushing's description as a 'third circulation', updated by Davson and Segal (1996), and nowmade current by ourselves and others (Haladky and Barrand, 2014; Spector et al., 2015; this review).

Human CSF also has multiple other functions for brain health. These include buoyancy for protection against blows to the head, transfer of micronutrients like certain vitamins and minerals from blood into CSF and thence into the ECSB, conveyance of large signaling and chaperoning molecules from blood (e.g., hormones) or synthesized in CP (e.g., transthyretin) into CSF, and removal of unnecessary molecules in brain (e.g., HVA, 5-HIAA or excessive amounts of Cu^{+}, riboflavin or nucleosides). CSF is also involved in the transfer bidirectionally (Schwartz and Baruch, 2012)of immunoregulatory molecules between brain and CP, with compensatory responses from CP via CSF (e.g., BDNF and IGF) stimulating the brain (Baruch et al. 2014) and helping it adapt to stress. Moreover, there may be streaming (guidance) processes for CSF in the lateral ventricle that transfer growth-promoting substances from CP to their proper targets – aided by ependymal ciliary beating (Sawamoto et al., 2006). This streaming hypothesis concept must be reconciled (integrated) with CSF mixing due to heart beat, respiration and posture in humans. In summary, like the kidney regulation of plasma composition and fluid dynamics, the CP-CSF flow axis has many diverseextracellular fluid functions that critically modulate brainfrom adulthood into aging.

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Table 1. Human CSF Composition – Selected Examples

1) Ions

a) Na^{+}, Cl^{-}, HCO_3^{-}, K^{+}, Ca^{++}, Mg^{++}, Mn^{++}

2) Vitamins

a) Vitamin C, folate, thiamine monophosphate, pyridoxal phosphate

b) Paradoxical transport (riboflavin, nucleosides and Cu^{+})

3) Peptides and proteins – transported from blood

a) Leptin, prolactin and IGF-1

4) Peptides and proteins synthesized in CP; released into CSF

a) Transthyretin, IGF-2, BDNF

5) Other growth factors and brain maintenance substances

a) Small RNA (90 species not present in plasma)

6) Proteins that diffuse from blood through barriers as a function of size

a) Albumin and immunoglobulins

Table 2. Recent Advances in Understanding CSF Flows

1) Realization of CSF flowing bi-directionally through Aqueduct of Sylvius

a) Influence of heart-beat, posture and respiration

2) Net CSF flow in humans; ~0.4 ml/min (average)

3) Diurnal patterns of flow from 0.2 →1.0 ml/min - more at night

4) Renewed understanding of differences between H_2O exchange across barriers and the net production of CSF containing salts (catalyzed by carbonic anhydrase in CP but not BBB)

5) Putative streaming of CSF in lateral ventricles

a) Directed growth factor delivery to specific regions of brain

b) Role of motile cilia at ependymal apical membrane

6) Better understanding of hydrocephalus/treatment and prevention

a) Role of mutations in ependymal ciliary apparatus (axoneme dynein)

b) Role of mutations in ependymal gap junction proteins (cadherin)

Table 3. Methodological Considerations in Studying CSF Composition and Functions

1) Effects of anesthesia

a) On cerebral blood flow

b) On cerebral function

c) On CSF production and flow

d) On diurnal variation in CP blood flow and CSF secretion

2) Effect of drugs

a) Acetazolamide (Diamox) and other carbonic anhydrase inhibitors to curtail CP formation of fluid

b) Atriopeptin (atrial natriuretic peptide) and other neuropeptides to inhibit ion transport and CSF production at the blood-CSF interface

3) Autonomic discharge and stress factors

a) Effects of adrenergic agonists and blockers on CP blood flow and CSF production

4) Effects of temperature, blood gases, pH, and osmolality on CP blood flow and CSF production

5) Tissue culture of neural stem cells or barrier cells – dedifferentiation/emergence or loss of functions

6) Effects of instrumentation and body fluid redistribution

a) Inflammation and brain trauma

b) Interference with normal functions like inspiration and gravitational adjustments (e.g. microgravity)

c) Pressure injections (injectate volume or hydrostatic pressure effects)

7) Differences and similarities between human and rodent brain

a) Size

b) Neuron/glial ratios

c) Size and function of astrocytes in rodents versus humans

d) Function of microglia (e.g., NO-generating system)

e) Distance from depth of brain to CSF

a) ~0.5 cm in rabbits (0.01 kg brain)

b) ~1.5 cm in human (1.2 kg brain)

f) New neuron production in human adult brain versus rodents

g) CP size and shape in rodents and other mammals, versus humans

8) State of brain/CSF at end of experiments

a) CSF composition (osmolality; and titer of neurohormones and Na^+/K^+)

b) Possible alteration of CP and BBB homeostatic functions

9) Importance of pharmacologic and physiologic principles

a) Dose-response and randomized controls

b) Time course of drug uptake from plasma

c) Differences between CSF production and H_2O fluxes

10) Withdrawal of CSF and analyses

a) Effect of sampled CSF volume on CNS physiology

b) Differential effects of single vs. multiple (serial) CSF aliquot collections on CSF chemical composition

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