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The proton-coupled folate transporter (PCFT-SLC46A1) and the syndrome of systemic and cerebral folate deficiency of infancy: Hereditary folate malabsorption

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Abstract

The proton-coupled folate transporter (PCFT) is the mechanism by which folates are absorbed across the apical brush-border membrane of the small intestine. The transporter is also expressed in the choroid plexus and is required for transport of folates into the cerebrospinal fluid. Loss of PCFT function, as occurs in the autosomal recessive disorder “hereditary folate malabsorption” (HFM), results in a syndrome characterized by severe systemic and cerebral folate deficiency. Folate-receptor alpha (FRα) is expressed in the choroid plexus, and loss of function of this protein, as also occurs in an autosomal recessive disorder, results solely in “cerebral folate deficiency” (CFD), the designation for this disorder. This paper reviews the current understanding of the functional and structural properties and regulation of PCFT, an electrogenic proton symporter, and contrasts PCFT properties with those of the reduced folate carrier (RFC), an organic anion antiporter, that is the major route of folate transport to systemic tissues. The clinical characteristics of HFM and its treatment, based upon the thirty-seven known cases with the clinical syndrome, of which thirty have been verified by genotype, are presented. The ways in which PCFT and FRα might interact at the level of the choroid plexus such that each is required for folate transport from blood to cerebrospinal fluid are considered along with a basis for the different clinical presentations of HFM and CFD.

Keywords

Proton-coupled folate transporter; PCFT; SLC46A1; Reduced folate carrier; RFC; SLC19A1; Folate receptors; FRα; Folates; Folic acid; Methotrexate; Pemetrexed; Hereditary folate malabsorption; HFM; Cerebral folate deficiency; CFD

1. Introduction

The B9 family of folate vitamins plays a central role in mammalian biology by providing the one-carbon moieties required for the synthesis of purines, thymidylate and methionine; the

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synthesis of glycine from serine; as well as the downstream epigenetic processes for which the synthesis of S-adenosylmethionine is required (Tibbetts and Appling, 2010). Essential to the maintenance of folate sufficiency is its adequacy in the diet, its intestinal absorption, and its transport into systemic tissues and into extra-systemic compartments. These different steps necessary for folate utilization are mediated by three different folate transporters. The identification of these transporters and an understanding of the distinct mechanisms by which folates are transported across cell membranes have emerged from research over the past five decades. This involved investigators that approached folate transport from different biological perspectives. One group focused on the role membrane transport plays as a determinant of the activities of, and resistance to, antifolate chemotherapeutic agents. Another group of researchers was concerned with folate homeostasis, folate deficiency and the critical role of intestinal absorption in folate sufficiency. It was the merging of these interests, that evolved from studies on the mechanism of membrane transport of the new-generation antifolate, pemetrexed, that led to the identification of the proton-coupled folate transporter (PCFT) (Zhao et al., 2004b) (Qiu et al., 2006; Wang et al., 2004).

It is now recognized that there are three folate-specific transporters that account for the transport of folates and antifolates. These are (i) the reduced folate carrier (RFC), ubiquitously expressed and the major and essential route of delivery of folates to systemic tissues (Matherly and Hou, 2008; Zhao and Goldman, 2013); (ii) PCFT, the mechanism by which folates are absorbed across the apical brush-border membrane of the proximal intestine, and essential to the transport of folates across the choroid plexus (Qiu et al., 2006; Zhao and Goldman, 2013); and (iii) the folate receptors, folate receptor alpha (FRα) normally expressed only in epithelia where it plays a role in trans-epithelial transport processes, and FRβ expressed largely in hematopoietic tissues (Elnakat and Ratnam, 2004;Kamenand Smith, 2004; Ross et al., 1994; Zhao et al., 2011a). This review will focus on PCFT, its functional characteristics, what is known about its structure/function and regulation, and the unique form of systemic and cerebral folate deficiency that occurs when there are inherited mutations in the PCFT gene that cause the loss of expression or function of its protein – hereditary folate malabsorption (HFM). In this review, folates will be used as a generic term for the B9 vitamins. Insome cases the folate will be specified: 5-methyltetrahydrofolate (5-methylTHF), the major folate inman; 5-formyltetrahydrofolate (5-formylTHF); and folic acid.

2. Distinguishing PCFT and RFC

RFC and PCFT are both members of the superfamily of solute carriers designated as SLC19A1 and SLC46A1, respectively. Both have secondary structures with twelve transmembrane domains (TMDs) with their N- and C-termini directed to the cytoplasm. The RFC gene is located on chromosome 21q22 while the PCFT gene is located on chromosome 17q11.2. RFC is a larger molecule consisting of 591 (NP_008927) versus PCFT's 459 (NP_542400) amino acid residues. Both human proteins are glycosylated in their first extracellular loop between the first and second TMDs, RFC at Asn66, and PCFT at Asn58 and Asn68, but glycosylation is not essential for their function. The composition of the two proteins is quite different with very low (~13%) sequence homology and, accordingly, there are marked functional differences. RFC is an organic phosphate antiporter that operates most

efficiently at pH 7.4. The adenine nucleotide gradient across the cell membrane, in particular, provides the energy source for the RFC-mediated uphill transport of folates into cells. PCFT, on the other hand, is a proton symporter that operates most efficiently at acidic pH; the proton gradient across the cell membrane provides the energy source for the uphill transport of folates into cells. The pH at the microenvironment of the proximal small intestine where folate absorption occurs is 5.8– 6.0 generated by sodium–proton exchangers in the apical membrane (Counillon and Pouyssegur, 2000;McEwan et al., 1990; Said et al., 1987). There are important differences in substrate specificity. RFC has a high affinity for the reduced folates, 5-methylTHF and 5-formylTHF (K_t ~ 1–5 μM), and a 10-fold higher affinity for PT-523, the δ-hemiphthaloyl-L-ornithine analog of aminopterin, but a very low affinity for folic acid (K_t > 150 μM). PCFT, on the other hand, has comparable affinity for folic acid and the reduced folates (K_m ~ 1– 3 μM) with no detectable affinity for PT523. This difference in substrate specificity, along with the difference in pH dependence, provides an approach to study the individual properties of PCFT and RFC, even under conditions in which both transporters are expressed (see next section). Neither carrier transports the polyglutamylated derivatives of folates or antifolates.

Both transporters are expressed in systemic tissues (Desmoulin et al., 2012; Whetstine et al., 2002). But location does not necessarily indicate a physiological role. It was the expression of RFC at the apical brush-border of the small intestine that led to the long-held assumption that this was the mechanism by which folates are absorbed across the apical brush-border membrane under physiological conditions despite the fact that the functional properties of folate absorption were very different from those of RFC-mediated transport and the pH in the microenvironment at the luminal surface is acidic and unfavorable for RFC function. Likewise, while PCFT is expressed in normal tissues its activity would be expected to be minimal at physiological systemic pH levels unless there were local areas of acidity generated by sodium–proton exchangers (Counillon and Pouyssegur, 2000). The properties of PCFT and RFC have been the subject of recent reviews (Hou and Matherly, 2014; Zhao and Goldman, 2013; Zhao et al., 2011a). Beyond the physiological role of PCFT, antifolates that are not substrates for RFC are being developed, but are selectively transported by PCFT within the acidic microenvironment of cancer tissues (Desmoulin et al., 2012). The properties of folate receptors are considered below within the context of their loss of function in cerebral folate deficiency.

3. Experimental approaches to the characterization of PCFT-mediated transport

The properties of PCFT and RFC can be largely isolated for transport studies by the pH employed: pH 5.5 for PCFT and pH 7.4 for RFC. However, under some conditions some folates/antifolates can have activities that extend into both transporters' pH range. This is particularly the case for the antifolate pemetrexed which sustains significant PCFT-mediated transport activity even at pH 7.4 (Zhao et al., 2008). Under conditions in which both carriers contribute to transport, the RFC-mediated component can be abolished selectively with a high concentration of PT523 and the PCFT component can be abolished selectively by a level of folic acid sufficiently high to block PCFT but insufficient to inhibit RFC-mediated

transport. When studying the properties of a unique PCFT mutant by transfection into cells, it is necessary to exclude the contribution of endogenous folate transporters. This can be achieved by using a cell line in which both activities are absent. For HeLa cells this was achieved by deletion of the RFC gene and silencing of PCFT expression due to methylation of the promoter under conditions in which folate receptors are not expressed (Diop-Bove et al., 2009; Zhao et al., 2004a). Hence, all constitutive folate transport activity is absent in this cell line (HeLa R1-11) so that all activity observed derives from the transfected PCFT construct. This is particularly important under conditions in which there is a low level of activity of the mutated PCFT so that its properties would be obscured by endogenous folate transport activity.

The properties of PCFT transport have been studied using a variety of different approaches and systems. Long before PCFT was cloned, folate transport using radiolabeled folate substrates was characterized in intestine in vivo, in intestinal sacs, in membrane vesicles derived from the apical membrane of intestinal cells and in cell lines of intestinal origin (Mason and Rosenberg, 1994). It was well known that the optimal pH for this process was acidic and that there was a high affinity for folic acid. It is now recognized that the activity observed in these studies was PCFT-mediated transport. Likewise, the low-pH folate transport activities detected in membrane vesicles derived from the basolateral membrane of hepatic cells (Horne et al., 1993), the apical membrane of renal tubular epithelial cells (Wani and Kaur, 2012), and the apical membrane of retinal pigment epithelial cells (Chancy et al., 2000) reflected PCFT-mediated transport. Since the genetic basis for this transport activity was clarified, the properties of this process have been studied by (i) transfection into mammalian cells and analysis of radiotracer fluxes, preferably under conditions in which there is no endogenous folate transport activity; (ii) expression in Xenopus oocytes by injection of the cRNA – a condition in which the only transport activity expressed in the otherwise dormant oocyte membrane is due to the expressed protein. Transport in oocytes is studied either with radiotracers or with electrophysiological measurements made under patch-clamp conditions in which the trans-membrane voltage can be adjusted (Nakai et al., 2007; Qiu et al., 2006, 2007; Umapathy et al., 2007). PCFT-mediated transport is electrogenic which reflects the movement of net positive charge(s) across the cell membrane. The electrogenicity requires cotransport of protons in excess of the negative charges carried by the folate molecule. Measurements in oocytes also allow the direct determination of changes in intracellular pH which reflects the transport of protons (Unal et al., 2009a). Intracellular pH measurements can also be made in cell lines, and correlated with folate transport, using pH-sensitive dyes as has been applied in HeLa cells (Zhao et al., 2013).

The highest level of low pH folate transport activity in mammalian cells has been detected in Caco-2 cells derived from a human colon cancer (Subramanian et al., 2008b). This reflects almost exclusively transport mediated by PCFT since more than 80% of the activity was abolished when PCFT mRNA expression was suppressed first by stable transfection of Caco2 cells with an shRNA construct followed by transient transfection with a siRNA duplex (Qiu et al., 2006). Caco2 cells can be grown as a tight monolayer of differentiated cells, with high electrical resistance, on a membrane that separates two aqueous compartments. In this way PCFT-mediated bidirectional fluxes and net transport of folates

from the apical to basal compartments, as occurs in the intestine, can be measured under a variety of conditions (Thakur et al., 2015; Urquhart et al., 2010). These types of studies can also be performed with the MDCK canine kidney cell monolayers (Urquhart et al., 2010). These systems also provide a model for the assessment of the specific targeting and trafficking of the protein in a polarized system, as reported for the apical targeting of PCFT in kidney cells (Subramanian et al., 2008a).

4. Establishing the secondary structure of PCFT

Topological analyses of the PCFT protein within the cell membrane have utilized the substituted cysteine accessibility method. In this approach, residues are exchanged with Cys and, if function is retained, the accessibility of the Cys-substituted residue is assessed by biotinylation with the sulfhydryl-reacting, membrane impermeant, MTSEA-biotin (Zhao et al., 2010). If the Cys-substituted residue is biotinylated, it is in a region accessible to the extracellular compartment – either an external loop or a residue within the aqueous translocation pathway. If the Cys-substituted residue is not accessible, it is located intracellularly or within a transmembrane domain. If located within a transmembrane domain, the Cys residue should become accessible after the membrane is permeabilized or when a membrane permeable reagent is used. If biotinylation is blocked with a folate substrate, this suggests that the residue is located in or near the folate finding pocket. This approach requires that the MTSEA-biotin not react with an endogenous Cys residue. There are seven Cys residues in the PCFT protein; fortunately, when all seven Cys residues are replaced with Ser, PCFT expression and function is preserved (Zhao et al., 2010).

Using this methodology, along with HA tagging of either the N- or C-termini, twelve transmembrane helices with N-and C-termini located in the cytoplasm were defined for PCFT after transfection into HeLa cells as illustrated in Fig. 1 (Qiu et al., 2007; Unal et al., 2008; Zhao et al., 2010). Subsequent studies extended the number of residues that were Cys substituted thereby further confirming and refining the topology (Date et al., 2016; Duddempudi et al., 2013). In the latter studies, expression and accessibility of the Cys-substituted residues was assessed in oocytes. In the studies with HeLa cells and oocytes, the second intracellular loop between the 2nd and 3rd TMDS was localized to the intracellular compartment (Date et al., 2016; Zhao et al., 2010). This is at variance with another report in which residues within this loop in HeLa cells were found to be accessible leading the authors to suggest that this represents a reentrant loop that moves outward within the translocation pathway sufficiently during the transport cycle to make it accessible for biotinylation (Wilson et al., 2014). The basis for this discrepancy among these results is unclear. Topological analyses predict breaks in four of the PCFT transmembrane helices: TMD 1 – at Gly40/Pro41; TMD 4 – at Gly158/Gly159; TMD 8 – P314; and TMD 10 – Gly361/Tyr362/Gly363. These breaks likely play an important functional role in the oscillation of the carrier between its inward-open and outward-open conformational states (Fig. 1).

While PCFT function can be fully preserved in the absence of all Cys residues, two residues were found to form a disulfide link: Cys66 in the first extracellular loop and Cys 298 in the 4th extracellular loop (Zhao et al., 2010); this linkage is not required for function. Cys-less

PCFT is, however, fragile; its stability and function can be disturbed when an additional mutation is inserted into the protein. For example, introduction of a K422C or K422E mutation into wild-type PCFT does not alter expression or function, but the protein is not expressed when these constructs are transfected into a Cys-less PCFT (Zhao et al., 2011b). Interestingly, as Cys residues are re-introduced into the protein, stability improves, a phenomenon that is related largely to the number of Cys residues, rather than the specific residues introduced (Zhao et al., 2011b). There is evidence that PCFT exists as a homodimer; however, this does not appear to have functional consequences (Hou et al., 2012; Zhao et al., 2012).

5. Modeling the tertiary structure of PCFT

At the present time only a few crystallographic structures of eukaryotic solute carriers have been obtained (Coleman et al., 2016; Deng et al., 2014, 2015; Nomura et al., 2015; Pedersen et al., 2013; Penmatsa et al., 2013). However, there are numerous structures of prokaryotic transporters that share homologous protein folds with eukaryotic transporters. This has provided the basis for the computation of three-dimensional comparative protein structure models. The most suitable template for the construction of a three-dimension model of PCFT has been the glycerol-3-phosphate (GlpT) *Escherichia coli* transporter (Huang et al., 2003). This model (Fig. 2) has been employed to better understand the role residues and domains play as determinants of PCFT function (Date et al., 2016; Lasry et al., 2008; Shin et al., 2012b, 2013; Unal et al., 2009a; Visentin et al., 2015; Zhao et al., 2012). Modeling has been particularly useful for the prediction of potential interactions between and among transmembrane domains and between residues. For instance, the GlpT structure, determined in the inward-open conformation, was used to predict the transmembrane domains that come together to form an extracellular gate for PCFT and residues within these TMDS that might be in sufficient proximity to form a disulfide bond either spontaneously or in the presence of an agent that promotes cross-linking. In this way, an external gate was confirmed by disulfide cross-linking of Cys-substituted residues, involving TMDs 1, 2, 7 and 11 (Zhao et al., 2016). The homology model also illustrates some of the breaks in the transmembrane helices.

6. The role of protons in PCFT-mediated transport

Proton coupled transport is typical of solute carriers expressed in the proximal small intestine that mediate the absorption of a variety of organic nutrients and ions (Anderson and Thwaites, 2010). Unlike the peptide transporters that accept a spectrum of organic structures, PCFT has a high degree of specificity for its monoglutamyl folate substrates. Co-transport of protons is critical to PCFT function; as the pH is increased, transport decreases due to an increase in the influx K_t and a decrease in V_{max}. However, this is substrate-specific; for instance, small changes in these parameters, as compared to other folates/antifolates, allow retention of significant PCFT-mediated transport of pemetrexed even in the absence of a pH gradient (Zhao et al., 2008). Transport occurs through a cycle of ordered folate and proton binding to, and dissociating from, the carrier. Fig. 3, cycle A, illustrates folate and proton cycling mediated by the carrier when a pH gradient is present. Following the alternating access model (Guan and Kaback, 2006; Stroud, 2007) the uptake cycle starts with the carrier

in an outward facing conformation. In the step 1, proton binds to PCFT resulting in an allosteric conformational change in the binding pocket that increases the affinity of the carrier for its folate substrate. In step 2 the folate binds to the carrier following which the carrier goes into an occluded state. In step 3, the carrier then shifts to an inward facing conformation. In step 4 folate is released, and in step 5 proton is released into the cytoplasm. In step 6 the carrier shifts to the outward-open conformation and the cycle is completed. For the LacY bacterial transporter, deprotonation is the rate-limiting step; the major change that occurred when proton-coupling was blocked by mutating a key glutamate residue was a marked decrease in the maximum influx rate (V_{max}) without any change in the concentration required to achieve half of the maximum rate, K_t, an indication of the affinity of the carrier for its substrate (Carrasco et al., 1989). Experimental insights have been obtained on the PCFT residues involved in these processes. The sole kinetic change observed for the E185A PCFT mutant at pH 5.5 was a marked decrease in the influx V_{max}; there was no change in influx K_t. Folate binding was unaffected by this change. However, influx mediated by the E185A mutant was fully preserved at pH 7.4 in the absence of a pH gradient, consistent with a defect solely in the proton coupling mechanism (Unal et al., 2009b). Hence, Glu185 is required for proton-coupling.

Fig. 3, cycle B, illustrates the transport cycle at a neutral pH in the absence of a pH gradient. Under these conditions, the binding step only involves the folate molecule and the carrier, and the rate-limiting step in the cycle is the rate at which the unloaded carrier alternates between the inward- and outward-facing conformations. Evidence that this is the case comes from the demonstration that folate influx is accelerated when the conformational change from the inward to outward conformation (efflux) occurs with a folate substrate in the binding pocket – trans-stimulation. Accordingly, while trans-stimulation of folate influx does not occur with wild-type PCFT at pH 5.5, when cycle A is dominant and the rate limiting step is de-protonation, it does occur at this pH for the E185A mutant, when a pH gradient and coupling is absent, and transport is now mediated via cycle B (Unal et al., 2009b).

While the Glu185 residue is required for proton-coupling, the His281 residue is required for proton binding (Unal et al., 2009a). When His281 was mutated to Ala281, there was a marked (10-fold) increase in the influx K_t at pH 5.5, consistent with a 10-fold *decrease* in the affinity of the carrier for its folate substrate. There was no significant decrease in the influx V_{max}. However, when the proton concentration was increased to 5.0 and 4.5, there was a marked (>10-fold) increase in folate transport mediated by the H281A mutant, and a decrease in the influx K_t while influx mediated by wild-type PCFT was essentially unchanged. Hence, His281 appears to play an important role in proton binding which, in turn, allosterically modulates folate binding. When the H281 residue was mutated to Ala, the affinity for proton decreased, i.e. the proton binding constant increased resulting in the increase in folate influx K_t. When the proton concentration was increased, this compensated in part for the reduced affinity for protons, and partially restored folate binding and transport. Evidence that His281 is not required for proton-coupling comes from electrophysiological studies in Xenopus oocytes demonstrating that folate transport mediated by the H281A mutant retains the capacity for cellular acidification (Unal et al., 2009a).

The interaction between protons and proton-coupled transporters often involves more than one residue. In the case of PCFT, no residue other than Glu185 has been demonstrated, as yet, to be involved in this process. However, more than one residue appears to impact on proton binding. Of particular interest is Arg376, a residue found to be mutated in several patients with HFM, as discussed below (Mahadeo et al., 2010). For the R376Q residue, identified in one patient, there was a marked increase in the influx K_t for reduced folates along with a decrease in V_{max}. This appeared to be substrate-dependent since for the antifolate, pemetrexed, the change was due solely to an increase in the influx K_t when function was assessed in HeLa cells that lack endogenous folate transporters. Further, pemetrexed transport was equivalent to that of wild-type PCFT when the pH was decreased to 4.5 for the R376Q, suggesting a role for this residue in proton-binding. Hence, the Arg 376 residue appears to play a role in proton, and consequently, folate binding.

7. Residues and domains critical to PCFT function and that define the aqueous translocation pathway

A number of approaches have been used to explore PCFT structure-function. These include random mutagenesis, site-directed mutagenesis, the substituted cysteine accessibility method and analyses of the functional properties of mutant forms of PCFT that have been identified in subjects with HFM. Initial interest was focused on conserved, charged residues. Among the negatively charged residues, Asp109 was found to be expressed but was irreplaceable, absolutely essential for function; Glu185 was found to have an important role in proton coupling (Shin et al., 2010; Unal et al., 2009b). Among positively charged residues, His247 and His281 were identified as playing an important role in function, the latter in proton binding.

His247 is fully conserved in a region that is usually poorly conserved (the large internal loop separating six TMDs on either side). When H247 was mutated there was an unusual pattern of change (Unal et al., 2009a). For instance, while the opposite charged Glu produced only a small decrease in function, the like-charged Arg substitution produced the greatest decrease in function. Influx kinetics with the H247A mutant indicated a large decrease in the folic acid influx K_t and V_{max}. While the level of protein expression of all mutants was comparable to that of wild-type PCFT, they all had a lower molecular weight and produced a narrower band on Western block consistent with impaired glycosylation (Unal et al., 2008). The homology model predicted that this residue comes into hydrogen bond distance with another conserved residue, Ser172 in the 2nd intracellular loop. When this residue was mutated to Ala, there was also a marked fall in the influx K_t, with a modest decrease in V_{max}. Also, while there were differences in K_i's among the reduced and oxidized folates for wild-type PCFT, the values dropped and became equal for all irrespective of the charge, size, or polarity of the His247-substituted residue. Finally, proton transport, independent of folate transport, was increased for the H247A mutant. The data suggested that H247, unrelated to its charge, perhaps in its interaction with S172, occupies a location that impacts on folate and proton access to the translocation pathway and the folate binding pocket. When this function is disturbed, by replacement with specific residues, folate binding is increased, but selectivity is lost, and proton transport independent of folate transport is increased, i.e. the

aqueous pathway now acts within this context as a “channel” for protons (Unal et al., 2009a).

Beyond the His residues, another positively charged residue required for function is Arg113 mutated in two subjects with HFM (Lasry et al., 2008, 2009; Zhao et al., 2007) and, along with Asp109, is found in the 2nd intracellular loop between the 2nd and 3rd transmembrane helices. Gly112, also required for function, is located in this loop (Wilson et al., 2014). Mutations in this region were reported to affect targeting of the PCFT protein to the apical membrane of Caco-2 and MDCK cells in monolayer cultures (Subramanian et al., 2008a). A subsequent analysis based upon homology to the GlpT suggested that the R113 residue may be embedded in a hydrophobic pocket formed by several TMDs (Lasry et al., 2008). Hence, with three residues essentially irreplaceable, it is clear that this loop plays a critical role in carrier function. While it has been proposed that this represents a re-entry loop, there is conflicting data on whether this domain has periodic access to the aqueous translocation pathway (Date et al., 2016; Wilson et al., 2014; Zhao et al., 2010). Although the C-terminus often contains a targeting motif, deletion of the C-terminus of PCFT did not alter targeting to the apical membrane of these cells (Subramanian et al., 2008a).

8. Regulation of PCFT expression

Folate deficiency results in an increase in PCFT mRNA expression; however, functional changes in transport are modest despite the crucial role of intestinal absorption in the maintenance of folate sufficiency. In the first study in which PCFT expression was assayed, for 8 weeks mice were fed a folate-deficient diet containing sulfathiazole to inhibit bacterial folate production. A 13.5-fold decrease in the blood folate level over this interval was accompanied by a comparable increase in PCFT mRNA expression in the proximal small intestine; however, function was not assessed in intestinal tissue (Liu et al., 2005; Qiu et al., 2007). More recently (Thakur et al., 2015), a folate deficient diet for 3–5 months in Wistar rats, in the absence of a sulfonamide, produced a lesser decrease in serum folate but a 5-fold increase in PCFT mRNA expression accompanied by a ~50% increase in the folic acid uptake V_{max} into intestinal brush-border membrane vesicles. While folate deficiency in Caco2 cells in vitro produced an 11-fold increase in PCFT mRNA, this was accompanied by a much smaller (~65%) increase in the apical to basal flux across a Caco2 monolayer (Thakur et al., 2015). At the molecular level, changes in PCFT expression were not associated with alterations in the methylation of the promoter region as might be expected to occur with a methyl donor deficiency as a consequence of folate deficiency (Thakur et al., 2015). In an earlier study, much higher PCFT mRNA expression in villus versus crypt intestinal cells was reported but there was only a modest increase in PCFT protein and folic acid transport at low pH which accompanied confluency and differentiation of Caco2 cells (Subramanian et al., 2008b). These observations are generally consistent with earlier observations of only modest changes in intestinal folate transport activity at low pH under folate deficient conditions (Said et al., 2000; Wani et al., 2012).

There is some understanding of the regulation of PCFT at the molecular level. In HeLa cells, the basal promoter has been localized to 138– 157 bp upstream of the translational initiation site (Diop-Bove et al., 2009; Stark et al., 2009). There is evidence based upon in vitro

studies that nuclear respiratory factor 1, vitamin D and Kruppel-like factor play a role in PCFT expression (Eloranta et al., 2009; Furumiya et al., 2013; Gonen and Assaraf, 2010); this is described in more detail in a recent review (Visentin et al., 2014). However, the physiological role of these elements in the regulation of folate absorption is unclear. While vitamin D enhances PCFT expression in vitro, a ten day course of vitamin D in healthy human volunteers did not result in an increase in folic acid absorption or an increase in PCFT expression in biopsies of the jejunum (Kullak-Ublick et al., 2016). In another study, neither plasma nor hepatic folate levels were decreased in vitamin D receptor knock-out mice, nor were vitamin D deficient rat dams or their offspring folate deficient and, finally, vitamin D supplementation did not result in an increase in the folate blood level in healthy human subjects (Brandsch et al., 2014).

9. The intestinal absorption and enterohepatic circulation of folates

Dietary folates, largely polyglutamate derivatives of reduced folates, enter the proximal small intestine where they are converted to their monoglutamate forms by glutamate carboxypeptidase II at the apical brush border membrane (Darcy-Vrillon et al., 1988). Because of the fortification of food with folic acid, as well as the widespread use of folic acid containing vitamin supplements, a substantial portion of ingested folates are in the form of folic acid monoglutamate. The pH in the microenvironment of the surface proximal small intestine is in the range of 5.8–6.0 (Counillon and Pouyssegur, 2000; McEwan et al., 1990; Said et al., 1987), generated by sodium–proton exchangers, which favors the activities of both the carboxypeptidase and PCFT. Folic acid and the reduced folates are excellent substrates for PCFT under these conditions and are rapidly transported across the apical membrane into the enterocyte followed by export across the basolateral, serosal, membrane mediated by at least one member of the multidrug resistance-associated proteins (MRPs) (Kitamura et al., 2010). RFC is also expressed at the apical membrane throughout the small intestine (Wang et al., 2001). However, because RFC's pH optimum is 7.4, there is negligible or no activity at the absorptive surface. The lack of a contribution of RFC to folate absorption under usual dietary folate loads is confirmed by impaired absorption and folate deficiency when PCFT is inactivated in HFM (see below). After efflux from enterocytes, folates ultimately enter the hepatic portal system and are delivered to the hepatic sinusoid where they are absorbed across the basolateral membrane of the hepatocyte. Basolateral membrane vesicles derived from hepatocytes have a high level of low pH folate transport activity consistent with a high level of PCFT activity (Horne et al., 1993). Hence, this could account for folate absorption across this membrane although the pH at the sinusoidal surface of that membrane is not known. On the other hand, two members of the organic anion transporter family, SLC21A6 and SLC 21A8, are also expressed at the basolateral membrane although they have lower affinities for folates than PCFT. It is unclear as to the extent to which these transporters contribute to the hepatic uptake of folates, in general, or 5-methylTHF in particular. However, polymorphorphisms of SLC21A6 impact on the clearance of intravenously administered methotrexate, and high expression of this carrier increases hepatic uptake (Trevino et al., 2009; van de Steeg et al., 2009). Within the liver, folates form polyglutamate derivatives, a reaction mediated by the enzyme folylpolyglutamate synthetase (FPGS). Because these congeners are not substrates for either

RFC or PCFT, and most polyglutamates are not substrates for the MRPs, they are retained and built to high level within hepatocytes. The liver has among the highest levels of expression of PCFT, comparable to proximal small intestine, in human and murine tissues and is the major storage depot for folates (Qiu et al., 2006, 2007).

Folates are mobilized from the liver first by hydrolysis to their monoglutamates mediated by a lysozymal conjugase (Shafizadeh and Halsted, 2007). This is followed by export across the biliary canalicular membrane mediated by ABCC2 and the ABCG2 for delivery via the bile duct back into the gut where they once again become available for re-absorption through the enterohepatic cycling mechanism (Chen et al., 2003; Kusuhara et al., 1998; Vlaming et al., 2011), or, the folate can be transported back across the basolateral membrane, where MRPs are expressed along with bidirectional transporters, into the hepatic vein and from there to the systemic circulation (Rius et al., 2003; Slot et al., 2011).

10. Hereditary folate malabsorption

Long before there was an understanding of the molecular identity of folate membrane transporters, a rare apparently autosomal recessive inherited disorder of folate malabsorption was recognized and named Hereditary Folate Malabsorption, HFM. In the first report by Lubhy et al. (1961) and in subsequent classical clinical investigative studies (Corbeel et al., 1985; Geller et al., 2002; Lanzkowsky, 1970; Lanzkowsky et al., 1969; Malatack et al., 1999; Poncz and Cohen, 1996; Poncz et al., 1981; Urbach et al., 1987), the major elements of this disorder were defined: (i) The failure to absorb an oral load of folic acid indicating an impaired intestinal folate absorptive mechanism; and (ii) Extremely low levels of folate in the cerebrospinal fluid (CSF) even after the folate blood level is normalized consistent with impaired transport of folate from blood across the choroid plexus epithelium into the CSF. Impaired intestinal absorption of folate results in very low folate blood levels with folate deficiency manifested systemically by severe macrocytic anemia which can be corrected with parenteral administration of low doses of folate. However, even when the blood folate level is corrected in this way, the CSF folate level remains very low and the normal, at least 2–3 fold CSF:blood folate concentration ratio, cannot be achieved. Over the years a clear picture of the clinical evolution of this disorder, and the spectrum of abnormalities that occur with this form of folate deficiency in infants, has been defined (Diop-Bove et al., 2014; Geller et al., 2002). Finally, the molecular basis for HFM was clarified with the discovery of PCFT and the identification of loss-of-function mutations in this gene in subjects with this disorder (Diop-Bove et al., 2014; Qiu et al., 2006; Zhao et al., 2007). To date there have been 37 families with a propositus with the clinical syndrome of HFM; 30 have genotypic confirmation (Table 1).

10.1. The clinical presentation

Infants with HFM come to medical attention several months after birth when they present with failure to thrive, developmental delays and anemia (Diop-Bove et al., 2014; Erlacher et al., 2015; Geller et al., 2002). There is often a clinical history of a sibling who died of unknown causes within a few months after birth. Often there can be gastrointestinal signs (diarrhea, stomatitis) reflective of the folate requirements of the proliferating intestinal

epithelium. However, despite the early presentation there can be long delays in diagnosis and treatment. Since the majority of cases are the products of consanguineous marriages, frequent in underdeveloped countries, delayed diagnosis is frequent in these settings. It is clear that the folate deficiency occurs soon after birth, certainly within one to two months, as established when younger siblings of older children with HFM are evaluated before there are very obvious clinical mani-festations of the disorder (Atabay et al., 2010; Geller et al., 2002). Some of the elements that result in a delay in diagnosis once anemia is recognized is a lengthy trial of oral folate, when the diagnosis is mistaken for a dietary folate deficiency, or intravenous administration of folate-containing vitamins that results in rapid, but transient, correction of the anemia. In the end, it is the rarity of the disorder, the lack of familiarity of physicians, and the spectrum of modes of presentation that confound and delay diagnosis. A description of the clinical presentation of subjects with HFM was summarized recently (see Supplementary Table 1) in Erlacher et al. (2015).

10.2. The anemia of HFM

Anemia is severe and often accompanied by leukopenia and thrombocytopenia. While usually macrocytic, it can be normocytic possibly due to the chronic nature of the illness and its infectious complications or an associated iron deficiency not related to impaired intestinal iron absorption. PCFT was initially reported to be a low affinity iron-heme transporter (Shayeghi et al., 2005); however, that activity has not been confirmed and there is no evidence that subjects with HFM or PCFT-null mice are iron-deficient (Qiu et al., 2006; Salojin et al., 2011). A unique aspect of this folate deficiency syndrome is an **immune deficiency** that has been reported in six subjects with HFM and is associated with infections such as CMV and, most typically, *Pneumocystis jiroveci* pneumonia. This always occurs in a setting in which there is marked hypogammaglobulinemia that is corrected rapidly with correction of the folate deficiency. The immune deficiency, studied in detail in recent years, involves defects in both B and T cell function. The immune deficient state has been reported in patients from diverse ethnic backgrounds: African-American (Malatack et al., 1999), Puerto Rican (Borzutzky et al., 2009), Central American (Zhao et al., 2007), Iraqi (Erlacher et al., 2015), Japanese (Kishimoto et al., 2014), and Turkish (Corbeel et al., 1985). One interesting aspect of the pneumonia is that it can progress rapidly after the administration of folate. While the reason for this is not known, it has been proposed that the immunological defects, sometimes with leukopenia, result in a failure tomount an inflammatory response to the infection which emerges after folates become available and the immunological defects are rapidly reversed (Erlacher et al., 2015; Kishimoto et al., 2014; Malatack et al., 1999). Be that as it may, with timely folate repletion and appropriate treatment, the pneumonia usually resolves. Infants with the immune deficiency of HFM can appear clinically, and after initial laboratory studies, very much like infants with combined immunodeficiency syndrome and are at risk of being managed as such. Hence, HFM must be considered and ruled out before a diagnosis of combined immunodeficiency syndrome is established.

10.3. Neurological aspects

Another major aspect of HFM is the neurological defects associated with this disorder. Beyond developmental delays, there are a variety of neurological manifestations: peripheral neuropathies, motor impairment, ataxia, behavioral disorders, cognitive defects and frank

mental retardation. This occurs in about one-third to half of the patients with HFM. The onset and severity vary from patient to patient and in the time to their appearance and intensity ranging from the time of presentation to several years. Ultimately, when left untreated, many patients develop seizures that may be severe and intractable. What is most pernicious about the neurological elements of this disorder is that they often occur or persist even after effective treatment of the systemic folate deficiency. After correction of the anemia, there is a tendency to consider treatment as adequate. However, the problem that remains is the magnitude of the defect in transport across the choroid plexus, the organ that transports folates uphill into the CSF against a concentration gradient. Hence, even when the folate blood level is normalized, the CSF folate level remains low in HFM. The CSF folate level necessary to sustain normal brain development has not been established. Clearly this is most critical in infants and young children for whom there is ongoing myelination and brain development.

10.4. Heterozygotes and fertility

Human heterozygotes have not been studied methodically; however, while there is probably diminished intestinal folate absorption and some decrease in blood folate, this does not appear to be clinically obvious. In PCFT +/− mice, in which this can be evaluated more precisely, there is a significant reduction (~30–50%) in intestinal folate absorption and blood and tissue folate levels (Salojin et al., 2011). Apparently, the fetal folate levels during gestation are sufficient since there have been no reports of neural tube, or other neurological defects in heterozygotes or PCFT-null infants, nor are there developmental defects in PCFT-null pups, at birth. Hence, placental folate transport to the fetus is sustained in the absence of PCFT and it is only after birth, with the loss of the maternal parenteral folate supply, that the folate deficiency state emerges. When PCFT-null mice are supplemented with adequate folate, they mature normally and are fertile (R. Fennell, personal communication). However, the only information regarding fertility in humans comes from a woman with HFM, diagnosed and treated within a few months after birth with parenteral 5-formylTHF, known to the authors (Min et al., 2008; Poncz and Cohen, 1996; Poncz et al., 1981), and who has subsequently delivered two normal infants. This patient’s mutation resulted in a stop codon within the first exon that eliminated the PCFT protein. Hence, a PCFT-null woman can deliver normal heterozygotes.

10.5. Treatment

Because HFM is a rare disorder, there have been no formal studies that provide an established, “evidence based”, regimen for optimal treatment. It is clear that the anemia, immune dysfunction and gastrointestinal signs can be cured with small daily doses of parenteral folate or large doses of oral folates. Those endpoints for treatment are definable and easily achieved. The major challenge is achieving adequate CSF folate and this requires careful monitoring of CSF levels until a satisfactory concentration is reached. Of importance is that normal CSF folate blood levels during infancy and early childhood are much higher than previously appreciated: ~100–150 nM over the first year decreasing to ~50–90 by five years, falling further to >60 nM during puberty (Ormazabal et al., 2011; Perez-Duenas et al., 2011; Verbeek et al., 2008). These levels are difficult to achieve in subjects with HFM even with extraordinarily high doses of parenteral folate (Torres et al., 2015).

There are no data that provide firm support for oral versus parenteral administration of folate. However, in one report even an oral dose of 20 mg/kg 5-formylTHF produced subnormal levels of CSF folate (Geller et al., 2002). In a recent study, a methodical attempt was made to correlate dose of parenteral 5-formylTHF with CSF folate levels (Torres et al., 2015). At an IM dose of 20 mg, which produced a serum folate of >261 nM the average CSF 5-formylTHF level was only 38 nM and over the patient's first two years of life the average level was 25 nM on lower doses, yet the child had what appeared to be normal development. More common and alarming are examples in which these subnormal CSF folate levels, sustained over many years, led to seizures and other neurological deficiencies later in childhood that responded to increased folate doses and higher CSF folate levels. However, even in the absence of frank neurological deficits, it is the subtle cognitive defects that warrant aggressive correction and maintenance of CSF folate at levels as normal as possible for the age to maximize the possibility that children with HFM will realize their full intellectual and neurological potential. There can be some improvement in the neurological defects associated with HFM when adequate CSF folate levels are achieved, despite long intervals in which the developing brain has been deprived of folate.

Taken together, the safest approach to treatment is parenteral folate, daily, titrated to achieve a CSF folate level within, or as close as possible to, the normal range. Folic acid should not be used to treat HFM. Folic acid binds tightly to folate receptors and presumably would inactivate receptors in the choroid plexus that are required for 5-methylTHF transport from blood to CSF (Kamen and Smith, 2004). The preferred substrate is, of course, 5-methylTHF, the major physiological folate found in blood and tissues that enters the brain rapidly through the blood–brain barrier (BBB) (Levitt et al., 1971; Pardridge, 2012; Wu and Pardridge, 1999); however, a parenteral preparation of this folate is not available. There is a 15 mg oral form of the active (6S) isomer of 5-methylTHF, levomefolic acid; while there is no experience in the treatment of HFM with this preparation, it would make sense to use it rather than 5-formylTHF if oral administration is required and cost is not prohibitive. Beyond this, oral and parenteral racemic 5-formylTHF is readily available and is widely used for the treatment of HFM. Preparations of the active (6S) isomer of 5-formylTHF are also available for parenteral use (levoleucovorin), eliminating the unnatural isomer. The advantage here is that the entire dose is the active isomer; this becomes important when the amount/volume of the injected folate is limiting. In one case, a 50 mg daily IM dose of Isovorin produced a CSF folate level of 50 nM, sufficient to eliminate seizures that occurred late, at age 5 1/2 years, in a child with HFM known to the authors.

10.6. The mechanism of intestinal absorption of folates when HFM is treated with high oral folate dosing

The fact that there is inadequate folate absorbed with usual dietary folate intake in HFM, when PCFT function is absent, is indicative of the inadequacy of other absorptive mechanisms, such as RFC, that are present in the intestine. However, with pharmacological dosing of folates, enough is absorbed to completely correct the systemic folate deficiency. The mechanism by which this occurs is not clear. Possibilities include transport mediated, inefficiently, by RFC in the proximal small intestine where there is an acid pH at the luminal surface, or absorption in the more distal regions of the small intestine where the pH is

optimal for this carrier. If RFC is the route then, clearly, a reduced folate is the appropriate choice in contrast to folic acid, since the former has a high, and the latter a very low, affinity for RFC. Favoring a role for RFC is the increased expression of this transporter in the murine intestine that occurs with folate deficiency (Liu et al., 2005). Other transporters are present in the intestine, such as OATP2B1-SLCO2B1 (SLC21A9), but have very low affinity for folates (Visentin et al., 2012).

10.7. The spectrum of mutations in HFM

Table 1 and Fig. 4 describe the current thirty known subjects with HFM for whom PCFT mutations have been identified. This includes four previously unreported subjects studied in this laboratory. The first external loop of PCFT between the 1st and 2nd TMDs is a high GC-rich region and is a prominent site of mutations in HFM particularly between p.65 and p.68 (c.194-c.204). Beyond this, mutations are distributed throughout the molecule primarily within or at the interface of transmembrane helices. Most of the mutations have been homozygous and private, the latter with three exceptions: (i) the G to A mutation at position 5882, homozygous in ten subjects of Puerto Rican heritage; (ii) the R376W mutation detected in two subjects (R376Q, in a third); (iii) Two subjects of Turkish descent living in Europe were studied, one recent and one genotyped on blood obtained in 1985 (Corbeel et al., 1985), with the same R113S mutations; it is unclear as to whether they are related. The mutation at 5882 is in the splice acceptor of intron 2 at the intron 2/exon 3 boundary, which results in the skipping of exon 3, an alternatively splice form, which does not traffic to the cell membrane. There is another earlier case report from Puerto Rico of a subject with the clinical syndrome of HFM but without genotypic confirmation (Santiago-Borrero et al., 1973). A Puerto Rico allele frequency study consisting of 3% of births revealed a carrier frequency of 0.2% island-wide and a frequency of 6.3% in the central province of Villalba, the likely site of a founder mutation (Mahadeo et al., 2011). Hence, this diagnosis should be strongly considered in infants of Puerto Rican heritage that present with anemia and failure to thrive within a few months after birth.

Most mutations result in a loss of the PCFT protein due to insertion- or deletion-related frame shifts or generation of stop codons. There have been several point in-frame mutations identified; some result in an unstable protein that is not expressed (G147R, D156Y, A335D, G338R). The R113C/S mutants, at the junction of the 3rd **TMD** and the 2nd intracellular loop, are expressed and traffic to the cell membrane, but function is markedly impaired (Lasry et al., 2008; Zhao et al., 2007). This residue is essentially irreplaceable (Lasry et al., 2009). In two cases the mutants were expressed and there was sufficient residual activity to assess the nature of the defect, particularly by using antifolates as substrates. The P425R mutant at the junction of the 5th extracellular loop and the 12th TMD is expressed but the level at the cell membrane is reduced. This residue does not tolerate a positive charge substitution but is functional with a negative, polar, or neutral substitution (Shin et al., 2012b). The loss of function was more pronounced for the reduced folates, 5-formylTHF and 5-methylTHF, as compared to methotrexate, pemetrexed and to a lesser extent folic acid. There was sufficient activity for the two antifolates to quantitate their transport kinetics. There was a 27% and 54% decrease in the influx V_{max} for methotrexate and pemetrexed, respectively. By comparison, there was a 7-fold greater decrease in the influx V_{max} for the

reduced folates as compared to methotrexate. There was a marked discrepancy in the changes in influx K_t/K_i. There was a prominent (5–9 fold) *increase* in this parameter for methotrexate, indicating a prominent *decrease* in the affinity of the mutated carrier for this substrate, but a 30% *increase* in the affinity for pemetrexed. This is consistent with a substantial difference in the way these antifolates bind to the carrier.

Expression of another residue mutated in HFM, R376Q, was modestly decreased but trafficked to the cell membrane and had sufficient residual function to assess its properties (Mahadeo et al., 2010). In this case, the magnitude of diminished transport of folic acid, the reduced folates and methotrexate in HeLa cells was comparable. However, there was substantial retention of function for pemetrexed for which the major defect was a 14-fold increase in the influx K_t/K_i without a change in influx V_{max}. Hence, at saturating concentrations, pemetrexed transport mediated by the mutant PCFT was equal to that of wild-type PCFT. Interestingly, when the pH was reduced to 4.5, transport of pemetrexed was equivalent to wild-type PCFT; however, transport remained very low at this pH when 5-formylTHF was the substrate, again consistent with the substrate specificity of this transporter. Two subjects with HFM have been identified with the R376W mutation, one reported (Zhao et al., 2007), the other a Native American not previously reported. The R376W protein is fully expressed but its function is markedly impaired (Mahadeo et al., 2010).

Several other point mutants that result in loss of the PCFT protein have been identified. A335D and G338R deep in the 9th TMD (Shin et al., 2011, 2012a) caused a loss of protein; expression of either residue was lost with a positively or negatively charged substitution. The level of expression at the cell membrane for various mutants generally correlated with function. However, there was an intrinsic loss of function for the G338C mutant that revealed an interesting transport phenotype. There was a marked decrease in the influx V_{max} and K_t but there was only a negligible change in the influx K_i. Hence, the binding constant was essentially unchanged while the concentration at which the carrier was half-saturated was markedly decreased. This observation is indicative of the complexity of the K_t determination and the fact that there are multiple steps in the transport cycle that can affect this value independent of a change in the binding constant. This discrepancy in the influx K_t/K_i is, however, a very unusual finding for this transporter and the only such example after analyses of the kinetic/binding alterations of many mutant PCFT residues.

11. The mechanisms by which loss of PCFT and FRα function result in cerebral folate deficiency

HFM results in cerebral folate deficiency associated with extremely low CSF folate levels. Loss of function mutations in FRα also result in cerebral folate deficiency with extremely low CSF folate levels. The latter is a rare autosomal recessive disorder that was designated “cerebral folate deficiency” (CFD). To date, there have been only 13 reports of CFD due to loss of FRα function (Al-Baradie and Chaudhary, 2014; Cario et al., 2009; Grapp et al., 2012; Perez-Duenas et al., 2010; Steinfeld et al., 2009). This is not to be confused with a variety of other disorders, unrelated to a specific folate transporter defect, that also result in

low CSF folate levels, and respond to treatment with 5-formylTHF (Perez-Duenas et al., 2011; Serrano et al., 2012). The difference between HFM and CFD is that the latter is unaccompanied by systemic folate deficiency; intestinal folate absorption is normal. Nor is there evidence of any obvious pathophysiological changes as a consequence of the loss of FRα activity in other organs in which the receptor is expressed. In the discussion that follows, "CFD" will refer to the inherited disorder that accompanies loss-of-function of FRα.

The clinical picture of CFD is different from HFM. Whereas HFM presents within a few months of birth, subjects with CFD are normal at birth and infancy present up to 2–3 years later only with neurological signs: developmental delays, ataxia, motor disorders, hypotonia, autistic behavior with or followed shortly thereafter, by seizures. The blood folate level is normal unless there is an accompanying unrelated folate deficiency (it is of interest that FRα-null mice have impaired renal reabsorption of folate) (Birn et al., 2005). The CSF folate level, untreated, is usually less than 10 nM and more often ≤5 nM. Patients with CFD respond to treatment with 5-formylTHF and, it would appear anecdotally, that higher CSF levels are achieved with lower folate doses administered orally (Cario et al., 2009; Perez-Duenas et al., 2010; Steinfeld et al., 2009).

It is clear that the defect in both disorders is at the level of the choroid plexus to explain the absence of CSF folate in the untreated setting. A major route of delivery of 5-methylTHF to the brain is arterial via the vascular endothelial BBB. This is distinct from the blood:choroid plexus epithelial cell:CSF barrier (Levitt et al., 1971; Pardridge, 2012; Redzic, 2011; Wu and Pardridge, 1999). RFC is expressed at the BBB (Wang et al., 2001) as is PCFT (unpublished observation); however, PCFT activity is minimal at physiological pH and the rate of arterial delivery is so great that there is little possibility that there could be a low pH within the microenvironment of the cerebral capillaries. Folate receptors are not expressed at the BBB. Limiting the net transport of folate across the BBB are MRPs expressed in the endothelial wall of the microvasculature of the brain (Li et al., 2013).

Folate delivery from the CSF into the brain occurs by passive diffusion, first across the ependymal layer of the ventricles and the surface of the brain followed by diffusion into the brain parenchyma. Hence, most brain tissue dependent upon the CSF for folate delivery is likely located in proximity to the ependymal layer (Blasberg et al., 1975; Pardridge, 2012). One reason the neurological deficit in HFM occurs much sooner than in CFD is that there are two elements in the neural folate deficiency: (i) low blood folate with inadequate folate delivery to the brain across the BBB and (ii) very low CSF folate levels and negligible to absent folate diffusion by that route. On the other hand, the normal blood folate levels when there is loss of FRα function allows normal folate delivery across the BBB while the folate deficiency due to low CSF folate levels impacts primarily on that portion of the brain in proximity to the CSF. The fact that CSF folate is so low in CFD, in the absence of FRα and with normal arterial delivery, indicates that there is insufficient folate transport across the BBB to reach and, alone, adequately supply the apical ventricular, and likely the inner subventricular, zones and diffuse into the CSF (Lehtinen and Walsh, 2011; Lun et al., 2015).

The choroid plexus consists of three units located in the lateral, third and fourth ventricles of the brain designed to maintain the volume, flow, and composition of the CSF. Beyond this, the choroid plexus provides a rich supply of a variety of growth factors to the CSF that circulate throughout the ventricular and spinal system and diffuse or are transported across the ependymal barrier (Lehtinen et al., 2011). This provides the nutrients and factors critical to support the niche for neuroepithelial and radial glial progenitors in the apical ventricular, and inner subventricular zones (Lehtinen andWalsh, 2011; Lehtinen et al., 2011). It would be expected that a robust supply of folate would be necessary to sustain the replication of these cells particularly during development.

At the microscopic and functional levels the choroid plexus consists of an epithelium that separates the capillary system at the basolateral membrane from the cerebrospinal fluid at the apical membrane. All three folate-specific transporters are expressed in the choroid plexus but their distribution is different (Fig. 5). PCFT is localized to the basolateral membrane of the murine choroid plexus epithelial cells (Zhao et al., 2009). This was not found to be the case for human choroid plexus (Grapp et al., 2013). FRα is located primarily at the apical membrane with a lower level of expression at the basolateral membrane (Kennedy et al., 2003; Patrick et al., 1997). RFC is expressed at the apical membrane (Wang et al., 2001). While RFC is a bidirectional transporter, its location favors transport into choroid plexus epithelial cells. Within cells, PCFT co-localizes with FRα in endosomes. This has been observed in rat choroid plexus Z310 cells (Grapp et al., 2013), Mü ller cells of the retina (Bozard et al., 2010) and HeLa cells (Zhao et al., 2009). A lesser degree of co-localization was noted in murine choroid plexus (Grapp et al., 2013).

How then to account for the observation that loss of *either* PCFT *or* FRα function results in the failure to transport folate across the choroid plexus into the CSF? Folate receptors mediate an endocytic process. These molecules are anchored in the cell membrane by a glycosylphosphoinositol domain. In the process of transport, folates bind tightly to the receptor (K_b in nM range); the receptor then invaginates into the membrane to form a vesicle that is released as an endosome into the intracellular compartment. As the endosome matures the intravesicular pH decreases, the folate dissociates from the receptor and can exit the intact endosome (Kamen and Smith, 2004). As indicated above, PCFT and FRα appear to co-localize to the endosomal compartment and it would seem logical that PCFT is the mechanism of export. Supporting this concept is the observation that PCFT augments FR-mediated uptake in HeLa cells (Zhao et al., 2009). However, FR-mediated transport persists in some cells that lack PCFT, and FRα-mediated transport is intact for antifolates that are very poor substrates for PCFT (Deng et al., 2009). It may be that there are alternate endosomal folate efflux pathways in some but not all tissues.

In a recent detailed analysis of folate transport in the choroid plexus, it was proposed that FRα shuttles folate, transcellularly, from the basolateral to the apical membranes (Grapp et al., 2013). This is consistent with a similar shuttling mechanism across the proximal renal tubule (Sandoval et al., 2004).When FRα was transfected into a choroid plexus cell line, multivesicular bodies made up of FRα-expressing late endosomes formed which released their contents at the apical membrane as exosomes into the CSF. When grown on a monolayer, FRα-mediated basolateral to apical 5-methylTHF transport was documented and

unchanged even when RFC and PCFT expression was reduced consistent with a transcellular process and the role of FRα in this context is independent of the other transporters (Grapp et al., 2013). Finally, when FRα containing exosomes were injected into the lateral ventricle of mice, uptake into brain parenchyma was demonstrated as well as uptake into astrocytes in separate in vitro studies. Taken together, this study has added to the understanding of the role that FRα plays in folate transport across the choroid plexus (Grapp et al., 2013). What is not defined is the basis for the critical role that PCFT plays in folate transport across the choroid plexus although it is proposed, and seems likely, that PCFT is required for the export of folates from exosomes into the CSF and PCFT-mediated export of folates from endosomes within choroid plexus epithelial cells (Grapp et al., 2013; Zhao et al., 2009). Whether PCFT, by mediating folate transport across the basolateral membrane of the choroid plexus, contributes to the intracellular folate level or vectorial flow, with RFC-mediating export across the apical membrane, is uncertain. While a sodium–proton exchanger was thought to be expressed at the basolateral membrane, that could provide a driving force for PCFT-mediated folate influx, recent studies place the exchanger at the apical membrane (Damkier et al., 2009). There can be marked decreases in CSF folate in conditions in which folate transporters should be intact such as the mitochondrial dysfunction that occurs in Kearns–Sayre syndrome that causes energy depletion (Ormazabal et al., 2011; Serrano et al., 2012). Hence, it is possible that impaired purine nucleotide production due to folate deficiency might also result in impaired mitochondrial function in the choroid plexus.

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Abbreviations

PCFT	Proton-coupled folate transporter
RFC	reduced folate carrier
5-formylTHF	5-formyltetrahydrofolate
5-methylTHF	5-methyltetrahydrofolate
HFM	hereditary folate malabsorption
CFD	cerebral folate deficiency
CSF	cerebrospinal fluid
BBB	blood–brain barrier.

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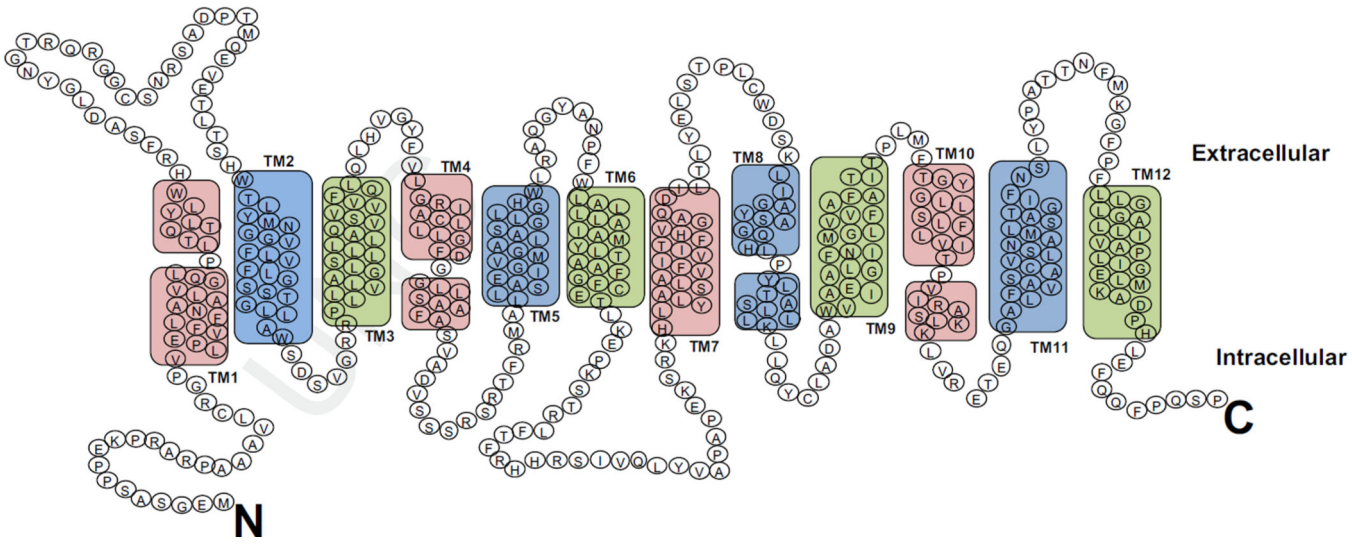

Fig. 1.

PCFT topology. The twelve transmembrane helices, six extracellular and five intracellular loops and the intracellular localization of the N- and C-termini were confirmed by the substituted cysteine accessibility method and hemagglutinin tagging (Qiu et al., 2007; Unal et al., 2008; Zhao et al., 2010). Breaks in the helices are predicted and shown for TMDs 1, 4, 8 and 10.

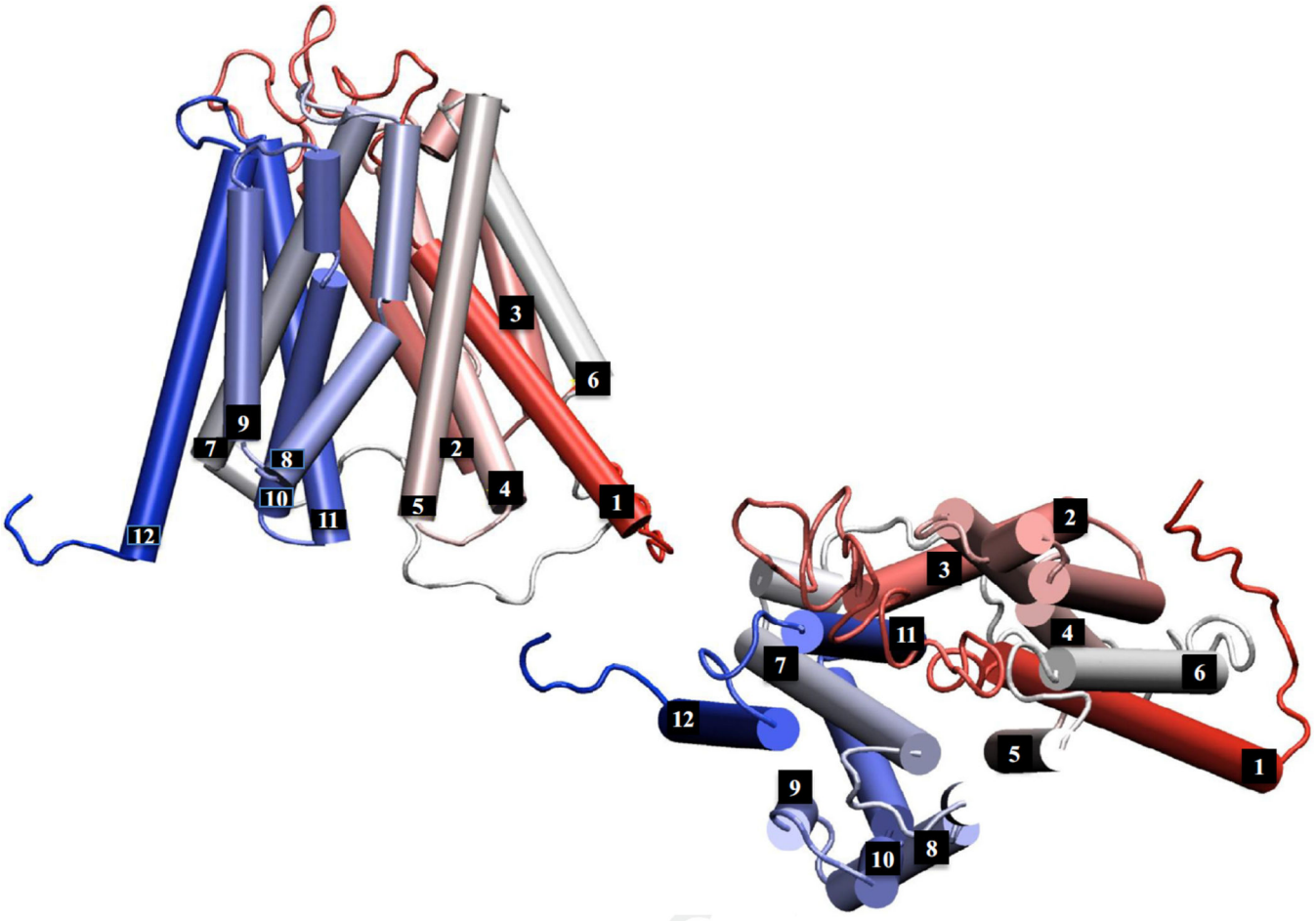

Fig. 2.

A homology model of PCFT in the inside-open conformation based upon the GlpT bacterial transporter (Huang et al., 2003). The transmembrane helices are numbered and colored going from red at the N-terminus to Blue at the C-terminus. Transmembranes 1, 2, 7 and 11 function as an extracellular gate (Zhao et al., 2016). Upper Left Panel: A lateral view of the model. Lower Right Panel: Looking into the aqueous translocation pathway from the extracellular compartment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

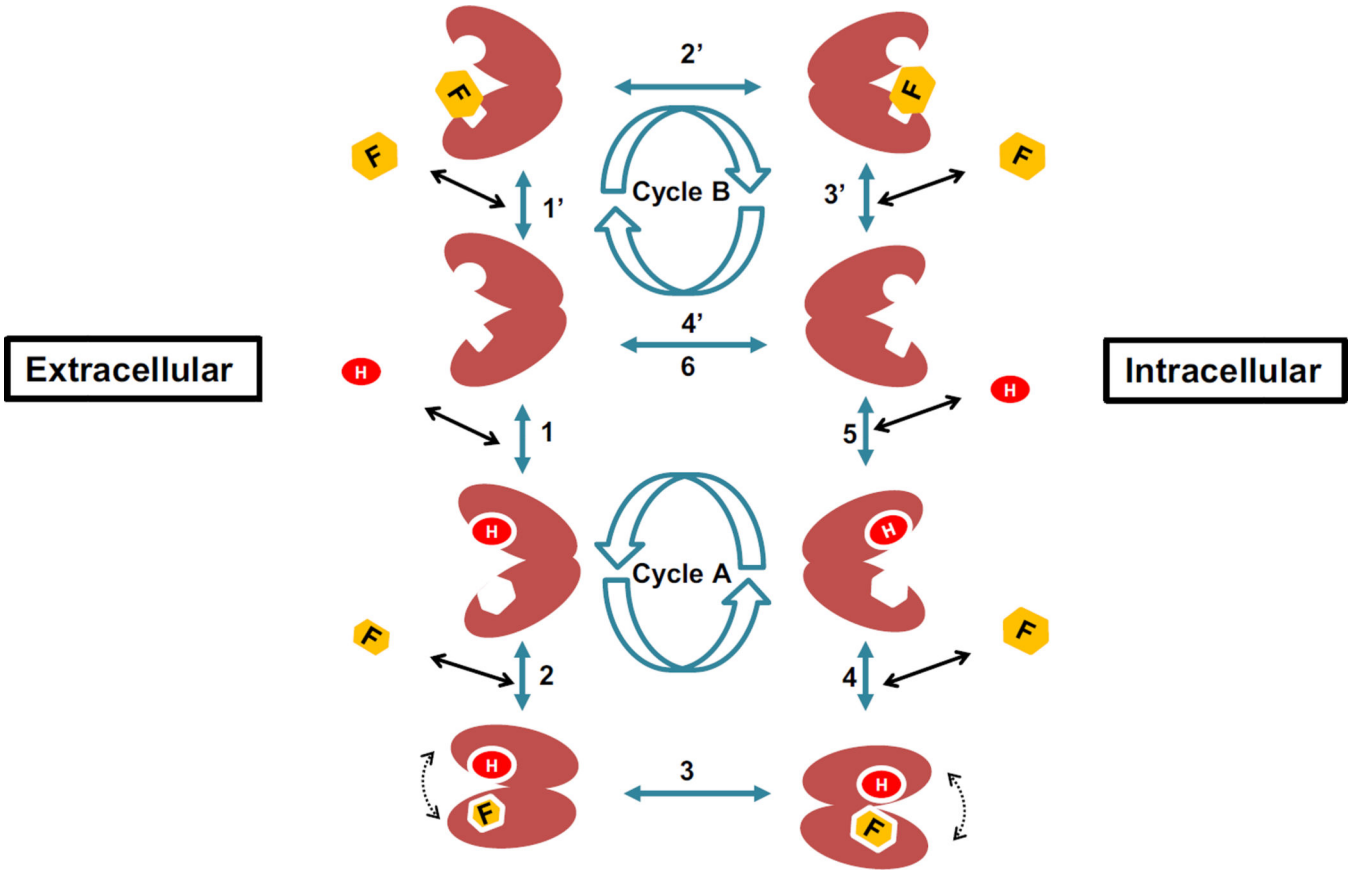

Fig. 3.

A model for the PCFT proton-coupling mechanism. Cycle A operates under conditions in which there is a transmembrane proton gradient. Association of protons, which are asymmetrically distributed across the cell membrane (out > in), with the carrier results, allosterically, in an increase in the affinity for folate substrate and an asymmetrical distribution of folate across the cell membrane (in > out). The rate at which proton dissociates from the carrier at the cytoplasmic interface (reaction 5) is rate-limiting. Cycle B operates in the absence of a proton gradient; the affinity for folate substrate is low and equal in the intracellular and extracellular compartments. Under this condition the rate at which the carrier changes its conformational state (reaction 4') is rate-limiting.

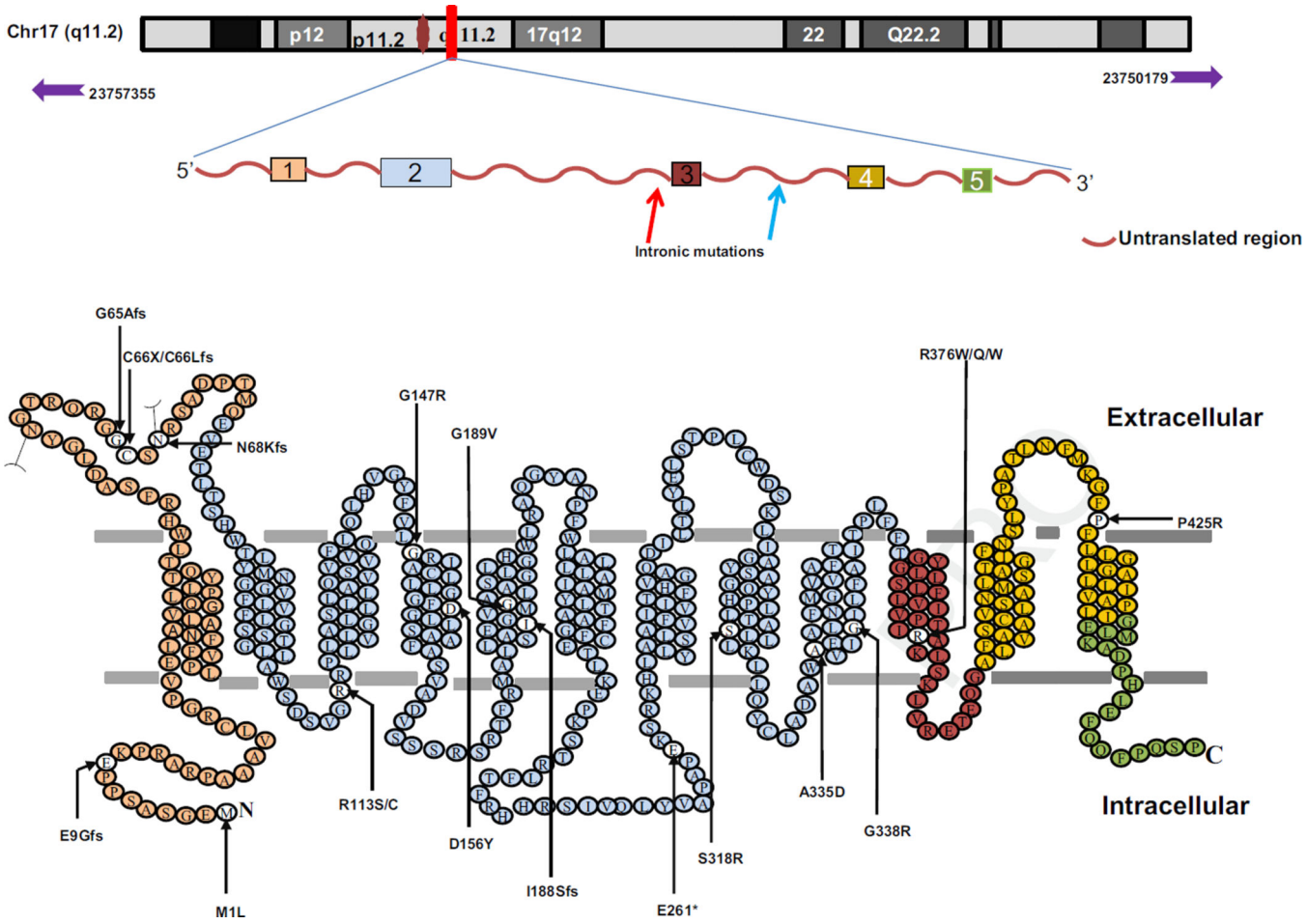

Fig. 4.

The organization of the PCFT genomic locus and the mutations identified in subjects with HFM. In addition, there are two mutations within introns. One in the spice acceptor site in intron 2 (red arrow) resulting in skipping of exon 3 (Qiu et al., 2006); the other a 168 bp insertion between exon 3 and 4 (blue arrow) leading to a premature termination (Kishimoto et al., 2014). Segments of the protein are color-coded to match the encoding exon. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

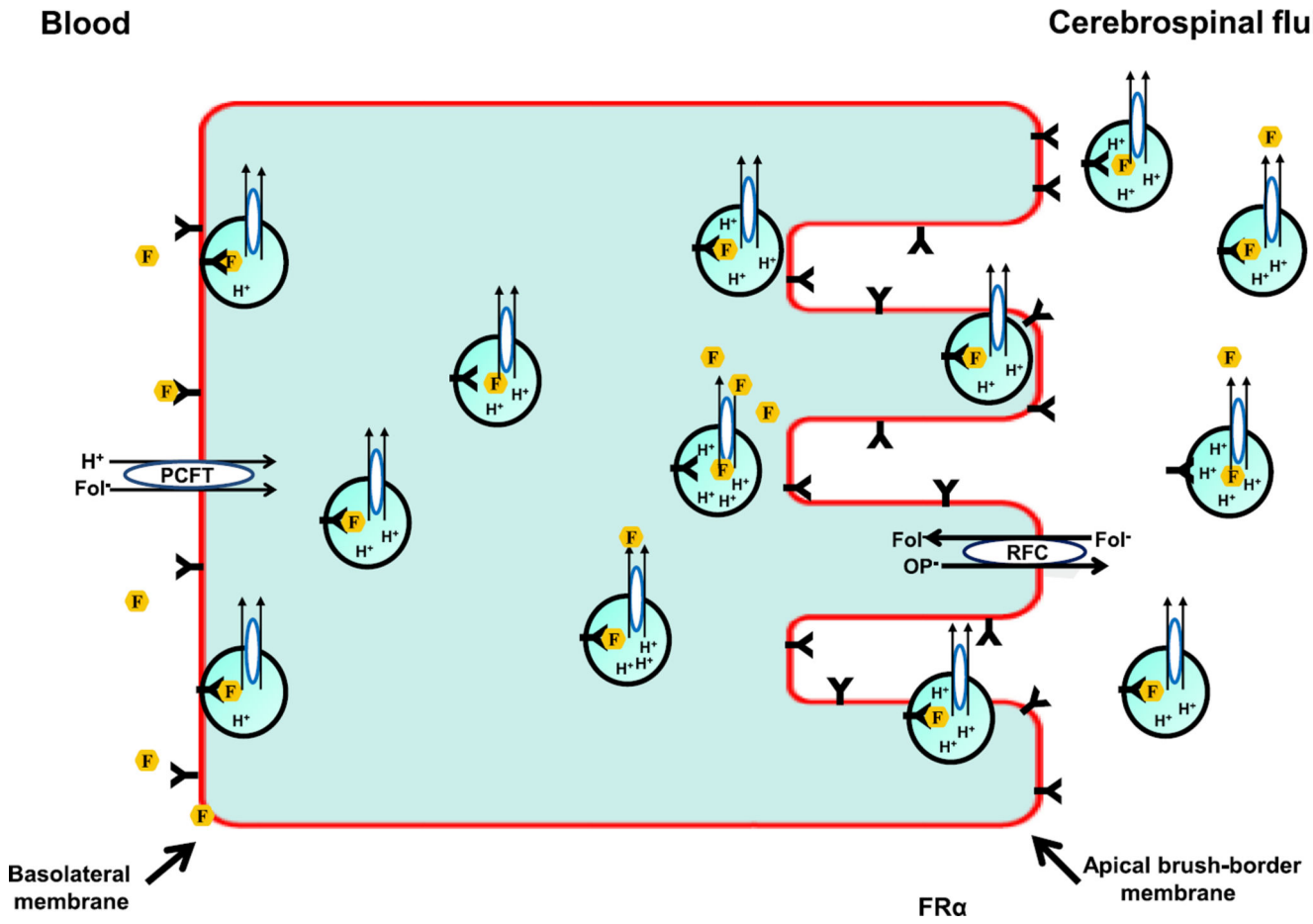

Fig. 5.

The location of folate-specific transporters in choroid plexus epithelial cells. PCFT has been located at the basolateral cellular and endosomal membranes. RFC is located at the apical brush-border membrane. FRα is located primarily at the apical membrane and, to a much lesser extent, at the basolateral membrane. One scheme to account for the requirement of both PCFT and FRα in the transepithelial flow of folate from blood to CSF is: a transcellular receptor-mediated mechanism whereby folate binds to receptor at the basolateral membrane where PCFT is expressed, a vesicle forms that contains both receptor and PCFT, traffics to the apical membrane where it is released into the CSF as an exosome from which folates are exported via PCFT (Grapp et al., 2013; Zhao et al., 2009). Endosomes also accumulate within multivesicular bodies within the cells that are discharged as exosomes into the CSF (Grapp et al., 2013). Alternatively, or complementing this, there is PCFT-mediated export of folates from acidified endosomes within the intracellular compartment followed by export into the CSF mediated by RFC, an organic phosphate (OP^{-}) antiporter (Zhao et al., 2009).

Table 1

Summary of PCFT mutations in subjects with HFM, listed by the chronological order of their identification.

Mutation in nucleotide	Mutation in protein	Ethnicity	Location at the time of study	Reference
c.1082-1G>A	p.Tyr362_Gly389del	Puerto Rican	Puerto Rico	(Qiu et al., 2006)
2009		Puerto Rican	Boston, MA	(Borzutzky et al., 2009)
2011		Puerto Rican	Puerto Rico	(Mahadeo et al., 2011)
2011		Puerto Rican	Rochester, NY	(Mahadeo et al., 2011)
2011		Puerto Rican	Worcester, MA	(Mahadeo et al., 2011)
2011		Puerto Rican	Puerto Rico	(Mahadeo et al., 2011)
2011		Puerto Rican	Puerto Rico	(Mahadeo et al., 2011)
2011		Puerto Rican	Puerto Rico	(Mahadeo et al., 2011)
		Puerto Rican	New York City	unpublished
		Puerto Rican	Hershey, PA	unpublished
c.194delG	p.G65Afs	African American	Pennsylvania	(Zhao et al., 2007)
c.337C>A	p.R113S	Turkish	Europe	(Zhao et al., 2007)
		Turkish	Strasbourg, France	unpublished
c.439G>C	p.G147R	Caucasian	Europe	(Zhao et al., 2007)
c.1274C>G	p.425R	Arab	Israel	(Zhao et al., 2007)
c.954C>G; c1126C>T	p.318R; p.R376W	Mexican	Houston, TX	(Zhao et al., 2007)
c.1126C>T	p.R376W	Native American	Minneapolis, MN	unpublished
c.337C>T	p.R113C	Arab	Israel	(Lasry et al., 2008)
c.197-198GC>AA	p.C66X	Portuguese	New Jersey	(Min et al., 2008)
c.1127G>A	p.R376Q	Chinese	Australia	(Mahadeo et al., 2010)
c.194dupG	p.C66Lfs	Pakistani	London, GB	(Meyer et al., 2010)
c.204-205delCC	p.N68Kfs	Turkish	Turkey	(Atabay et al., 2010)
c.466G>T	p.D156Y	Pakistani	Canada	(Shin et al., 2010)
c.1004C>A; c.204-205delCC	p.A335D, p.N68Kfs	English	London, GB	(Shin et al., 2011)
c.1012G>C	p.G338R	Tunisian/Arab	Tunisia/Arab	(Shin et al., 2011)
c.18-23insC	p.E9Gfs	Tunisian/Arab	Tunisia/Arab	(Shin et al., 2011)
c.558-588del	p.I188Sfs	Nicaraguan	Houston, TX	(Diop-Bove et al., 2013)
Not described; 168 bp intron insertion and c.1165-285T>G	p.G189V; premature termination	Japanese	Japan	(Kishimoto et al., 2014)
c.781G>T	p.E261X	Kurdish	Germany	(Erlacher et al., 2015)
c.1A>T; c.194-195insG	c.A1T (mutation of the initiation codon), p.C66L(fs)	Chinese	China	(Wang et al., 2015)