



HHS Public Access

Author manuscript

Dev Neurobiol. Author manuscript; available in PMC 2019 April 01.

Published in final edited form as:

Dev Neurobiol. 2018 April ; 78(4): 391–402. doi:10.1002/dneu.22579.

FOLATE ACTION IN NERVOUS SYSTEM DEVELOPMENT AND DISEASE

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Abstract

The vitamin folic acid has been recognized as a crucial environmental factor for nervous system development. From the early fetal stages of the formation of the presumptive spinal cord and brain to the maturation and maintenance of the nervous system during infancy and childhood, folate levels and its supplementation have been considered influential in the clinical outcome of infants and children affected by neurological diseases. Despite the vast epidemiological information recorded on folate function and neural tube defects, neural development and neurodegenerative diseases, the mechanisms of folate action in the developing neural tissue have remained elusive. Here we compiled studies that argue for a unique role for folate in nervous system development and function and its consequences to neural disease and repair.

Keywords

Folic acid; neural tube defects; folate receptor

INTRODUCTION

For more than 50 years epidemiological studies have demonstrated that periconceptional folate supplementation reduces the occurrence of one of the most common serious birth defects, called neural tube defects (NTDs). These defects manifest when the neural tube fails to close and form properly during the first four weeks of pregnancy (Wallingford et al., 2013). It is believed that neural tissue exposed to the amniotic fluid eventually degenerates (Meuli and Moehrlen, 2014), leading to severe neurological deficits that are lethal in anterior NTDs (anencephaly), or result in paralysis or motor disability when they occur at the spinal cord (spina bifida).

Strikingly, detailed understanding of the mechanisms of folate action during neural tube formation has remained elusive. Folate belongs to the vitamin B family and it participates in methylation reactions such as those necessary for nucleotide synthesis. Hence, folate supports rapid growth by enabling DNA synthesis in proliferating cells. It has been shown

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The authors declare that they do not have any conflict of interest.

that one of the cellular processes involved in normal formation of the neural tube in some vertebrates is oriented cell division (Schoenwolf and Alvarez, 1989; Schoenwolf and Yuan, 1995; Sausedo et al., 1997). This allows the developing neural tissue to acquire critical mass, and provides the preferred rostrocaudal orientation so that these divisions contribute to the elongation of the developing neural tube in chick and mouse embryos (Schoenwolf and Alvarez, 1989; Schoenwolf and Yuan, 1995; Sausedo et al., 1997; Keller, 2006). On the other hand, in other vertebrates like *Xenopus laevis*, the necessity for cell divisions during neural tube formation has been ruled out by demonstrating that blocking DNA synthesis does not interfere with neurulation (Harris and Hartenstein, 1991). Nevertheless, folate action is necessary during *Xenopus laevis* neural tube formation, as recently demonstrated by our study (Balashova et al., 2017), suggesting that folate might play additional functions during neural tube formation besides its role as a vitamin for DNA synthesis. Moreover, many knockout mice for enzymes directly involved in folate metabolism did not exhibit increases in NTD incidence (Watanabe et al., 1995; Chen et al., 2001; Swanson et al., 2001). In contrast, knocking out one of the folate uptake systems, folate receptor 1 (Folr1/folate binding protein 1/folate receptor α) leads to severe NTDs in mice (Piedrahita et al., 1999; Finnell et al., 2002; Spiegelstein et al., 2004; Wallingford et al., 2013). Similarly, mutations in folate uptake systems have been reported in families with higher incidence of NTDs than the general population, including 2 SNPs in FOLR1 (O'Byrne et al., 2010). These studies strongly indicate that folate acts as an essential regulator of neural tube morphogenesis, probably independent of nucleotide synthesis.

Here, we review the findings that address the role of folate in the development of the nervous system and folate's effects on neural function and repair. These studies argue for specialized functions of folate in neural tissue and propose new questions that require further investigation to be answered on the mechanisms of folate action in nervous system development, disease and regeneration.

FOLATE AND FOLATE UPTAKE SYSTEMS

Folic acid/folate (pteroyl-L-glutamic acid and pteroyl-L-glutamate, respectively) belongs to the B9 family of vitamins. Chemically, folic acid is a protonated form of folate anion present in solution; therefore the terms are used interchangeably. However, in the field of nutrition the term "folates" is frequently used to refer to the chemically diverse mixture of naturally occurring folic acid derivatives - pteroylglutamates. Pteroylglutamates may vary in one-carbon donor groups, degree of pteridine ring reduction and number of glutamate residues (O'Broin et al., 1975; Lewis et al., 1999).

In animals, folate cannot be synthesized *de novo*. Dietary folate is obtained in the form of dihydrofolate, tetrahydrofolate or 5-methyltetrahydrofolate, which are naturally present in a variety of foods, or as synthetic folic or folinic acid added to fortified food (O'Broin et al., 1975; Eitenmiller and Landen, 1999; Lewis et al., 1999). Folate is needed to carry one-carbon groups for methylation reactions and nucleotide base synthesis, and is therefore indispensable for DNA replication and repair, as well as RNA synthesis (Figueiredo et al., 2009). Folate is also involved in histone and DNA methylation, methionine production and homocysteine remethylation reactions (Stover, 2009). Thus, folates are especially important

during early embryogenesis, which is characterized by rapid cell divisions. In addition, folate is necessary for amino acid metabolism, neurotransmitter and phospholipid biosynthesis (Desai et al., 2016; Mentch and Locasale, 2016). Numerous clinical studies have established a correlation between low folate levels and increased risk of NTDs (Milunsky et al., 1989, 1991a; 1991b; Simpson et al., 1991), cardiovascular disorders (Bazzano et al., 2006; Wang et al., 2006; Wang et al., 2007), cognitive malfunction (Mitchell et al., 2014) and certain types of cancer (Kim, 1999; Sanjoaquin et al., 2005; Cole et al., 2007; Johansson et al., 2008) highlighting the importance of understanding the mechanisms of folate action.

Folates are hydrophilic molecules whose cellular uptake employs three transport systems: the proton-coupled folate transporter (PCFT), reduced-folate carrier 1 (RFC1), and folate receptors (FOLRs, also called folate binding proteins) (Matherly and Goldman, 2003; Kamen and Smith, 2004; Zhao and Goldman, 2007) (Table 1). PCFT is the main transporter for intestinal absorption of folate and plays a major role in folate homeostasis in humans (Zhao et al., 2009a; Zhao et al., 2009b). RFC1 is a typical anion antiporter that utilizes the gradient of organic phosphate across the cell membrane to transport folate into the cells at neutral pH (Goldman, 1971; Sirotinak and Tolner, 1999). FOLRs are high affinity folate binding proteins. In mammals, there are four known genes for FOLR. FOLR1, 2 and 4 are linked to the plasma membrane by a carboxy terminus - glycosylphosphatidylinositol (GPI) anchor (Kamen and Caston, 1986; Ratnam et al., 1989; Wang et al., 1992; Shen et al., 1994). FOLR1 is expressed in a wide variety of cell types and tissues including epithelial tissues (Weitman et al., 1992; Antony, 1996; Smith et al., 1999; Chancy et al., 2000). FOLR2 expression is restricted to hematopoietic tissues (Reddy et al., 1999). FOLR4 (also known as Juno) is found in the membrane of egg cells, where it facilitates fertilization via recognition of complementary sperm protein (Bianchi et al., 2014). FOLR3 is a secreted protein (Shen et al., 1995).

The field of cancer research has elucidated some aspects of the biochemistry and molecular function of folate transporters, since these transporters are often overexpressed in tumors and cancer cells, and have been used as conduits for anticancer drug therapeutics. From these studies we have learned that folate uptake by FOLRs and FOLR recycling in cancer-derived and immortalized mammalian cell lines occurs through endocytosis. The exact mechanisms of FOLR endocytosis vary depending on the experimental model used (Rothberg et al., 1990; Rijnboutt et al., 1996; Mayor et al., 1998; Kamen and Smith, 2004; Bhagatji et al., 2009; Elnakat et al., 2009). Acidification of the endosome interior to a pH of 6.5, as occurs in early endosomes, is required for dissociation of folate from FOLRs (Yang et al., 2007). Some studies support a model for FOLR1 internalization upon ligand binding through a Cdc42-regulated actin-dependent pathway, which is shared by the majority of GPI-anchored proteins (GPI-APs). Others have shown that FOLR1 is endocytosed in cholesterol and sphingolipid-enriched cell surface invaginations (Kamen et al., 1988) and once incorporated into vesicles, proceeds into GPI-AP-enriched early endosomal compartment (Mayor et al., 1998; Sabharanjak et al., 2002). This is followed by microtubular delivery to the recycling endosomal compartment via Rab7/dynein and Rab 4/kinesin I-dependent trafficking as demonstrated in KB and HeLa cells (Chen et al., 2008).

FOLRs subcellular localization also varies based on the cell type. FOLR1 is found in the apical cell surfaces of choroid plexus, placenta, brush-border membrane of proximal renal tubular cells and superficial neural plate cells (Weitman et al., 1992; Antony, 1996; Parker et al., 2005; Balashova et al., 2017) and basolateral membrane of retinal pigment epithelium (Smith et al., 1999; Chancy et al., 2000). Its GPI post-translational modification is found in organisms ranging from protozoans to mammals and serves to anchor a great variety of functionally different proteins to the outer leaflet of membranes (Lakhan et al., 2009). The polarized nature of FOLR1 subcellular localization is a characteristic of GPI-APs (Paladino et al., 2006), which are also known to be recruited to lipid rafts and to elicit downstream signaling triggered by GPI-AP endocytosis (Watanabe et al., 2007; Watanabe et al., 2009; Tassew et al., 2012). In addition, a number of GPI-APs have been shown to interact with transmembrane signaling molecules, including those involved in cell-cell and cell-matrix adhesion (Davy et al., 1999; Monnier et al., 2002; Paratcha et al., 2003; Matsunaga et al., 2004; Tassew et al., 2012). These properties of GPI-APs predict that FOLRs might play additional roles besides folate uptake.

FOLATE ACTION DURING NEURAL TUBE FORMATION AND IN NEURAL TUBE DEFECTS

The prevailing model for folate action during neural tube formation is centered on folate metabolism enabling nucleic acid biosynthesis, cell proliferation and growth. Knockout mice on some enzymes participating in folate metabolism support this model. For instance, mice lacking the mitochondrial monofunctional 10-formyl-tetrahydrofolate synthetase (Mthfd11) exhibit developmental delay and aberrant neural tube closure (Momb et al., 2013). The NTD phenotype is partially rescued by formate supplementation, suggesting that formate derived from mitochondria is necessary for adequate development and formation of the neural tube (Momb et al., 2013). Supporting the metabolic function of folate in neural tube defects, the incidence of NTDs is elevated in cases where either the infant or the mother are homozygous for methylenetetrahydrofolate reductase (van der Put et al., 1995; van der Put et al., 1996; Blom et al., 2006), although not every ethnic group studied exhibited this association (Papapetrou et al., 1996; Wilcken and Wang, 1996; Mornet et al., 1997; Speer et al., 1997; Koch et al., 1998). However, this model is thrown into question by mice in which several enzymes involved in folate metabolism are disrupted but do not show NTDs (Watanabe et al., 1995; Chen et al., 2001; Swanson et al., 2001), as well as screenings in humans that found cases with innate errors in folate metabolism which are not associated with increased incidence of NTDs (Blom et al., 2006).

One of the first folate-related mouse models of neural tube defects was the *Folbp1* (mouse FOLR1) knockout, which is embryonic lethal and associated with NTDs (Piedrahita et al., 1999; Tang et al., 2005), indicating that FOLR1 is necessary for neurulation. Although FOLR1 is able to bind folate with high affinity, transport through an endocytotic mechanism is less efficient for providing adequate levels of folate to the cell compared to the reduced folate carrier, as shown in leukemia cells (Sierra et al., 1995; Spinella et al., 1995). This suggests that folate transport may not be the primary function of FOLR1. At present the physiological role of FOLR1 in normal tissues is unclear, although disruption of *Folbp1*

gene results in misexpression of a number of essential signaling molecules, including upregulation of the ventralizing signal sonic hedgehog in abnormally expanded midbrain floor plate (Tang et al., 2005). Endocytosis of FOLR1 in the neuroepithelium appears to be dependent on the multifunctional endocytic receptor complex formed, among others, by Megalin, also known as LRP2. When LRP2 is knocked out, the neuroepithelium is deficient in folate and mice exhibit rostral neural tube closure defects (Kur et al., 2014).

The aforementioned studies strongly argue for a role of folate and FOLR1 in neural tube formation. Our recently published study was aimed at identifying the specificity of their function in terms of cellular processes regulated by folate action and the identity of targeted tissues dependent on FOLR1 role during neurulation (Balashova et al., 2017). We found that from the early stages of neural plate folding in *Xenopus laevis*, FOLR1 is expressed in superficial neural plate cells, where it localizes to the apical membrane. Similarly, in mouse embryos FOLR1 colocalizes with LRP2 in the dorsal neuroepithelial midline, which corresponds to the site where Closure 2 is initiated, and in the ventral midline of the midbrain neuroepithelium (Kur et al., 2014). Interestingly, knocking down FOLR1 in *Xenopus* neural plate cells only, elicits NTDs, while FOLR1 knockdown from non-neural ectoderm or mesoderm does not (Balashova et al., 2017), suggesting a specific requirement for FOLR1 in neural plate cells, essential for the formation of the neural tube.

We demonstrated that FOLR1 interacts with apically enriched components of adherens junctions like C-cadherin, the most abundant cadherin in the neural plate (Nandadasa et al., 2009), and β -catenin (Balashova et al., 2017), suggesting potential roles for FOLR1 in cell adhesion and cytoskeletal organization. One of the early and crucial cellular processes during neural plate bending is the apical constriction of neural plate cells, which occurs in frog, chick and mouse embryos, although at varying mediolateral and anteroposterior regions of the neuroepithelium. The Agarwala lab has demonstrated that the spatiotemporal regulation of apicobasal polarity in the folding neural plate by bone morphogenetic protein and transforming growth factor beta signaling drives the apical constriction of neural plate cells in the middle hinge point of neurulating chick embryos (Eom et al., 2011; Eom et al., 2012; Amarnath and Agarwala, 2017). Similarly, in frog embryos the medial and anterior localizations of planar cell polarity molecules at the neural plate midline are required for the changes in cell shape and movement necessary for neural plate folding and neural tube formation (Ossipova et al., 2014; Ossipova et al., 2015). In turn, neural cell apical constriction is postulated to be driven by the contraction of the actomyosin network anchored at the apical adherens junctions (Haigo et al., 2003; Nishimura and Takeichi, 2008; Martin et al., 2009). The precise dynamics of the proteins that form the adherens junctions during this process remain to be determined. Nevertheless, studies from the Sokol lab have demonstrated that Rab11-containing recycling endosomes accumulate in the apical adherens junctions of medial neural plate cells through a planar cell polarity- and Rho family GTPase GEF-H1-dependent mechanism, and this apical Rab11 localization is necessary and sufficient for apical constriction (Itoh et al., 2014; Ossipova et al., 2014). We discovered that FOLR1 is necessary for apical constriction of neural plate cells and C-cadherin endocytosis, which is apparent in constricting neural cells (Balashova et al., 2017); the necessity of this process becomes obvious when considering the dramatic reduction in cell apical surface occurring during apical constriction, which has to be accompanied by removal of the excess

of apical membrane and transmembrane proteins, including those mediating cell-cell adhesion (Fig. 1). Indeed, it has been shown that endocytosis is involved in apical membrane removal during contraction of cell apical surface in a number of model systems (Lee and Harland, 2010; Mateus et al., 2011). We propose that endocytosis of adhesion proteins facilitated by FOLR1 may be needed to enable reduction of apical cell circumference and redistribution of adhesion molecules from cell apices to lateral intercellular contacts to accommodate for dynamic cell shape changes during neurulation (Fig. 1). Interestingly, a model of neurulation named “cortical tractor” has been proposed in which recycling of cell adhesion proteins from the apical circumference to the cell basolateral surface is required for cell shape changes (Jacobson et al., 1986; Jacobson, 1994). More recently it has been demonstrated that collectively migrating astrocytes utilize a mechanism similar to the cortical tractor to recycle N-cadherin molecules (Peglion et al., 2014).

Our study (Balashova et al., 2017) suggests that the role of folate and FOLR1 during neural tube formation may not be related to its metabolic function as a vitamin (Fig. 2). Intriguingly, folate is a chemoattractant for the model unicellular organism *Dictyostelium discoideum*, in which it elicits changes in the cytoskeleton that enable the amoeba to crawl towards the food source (McRobbie and Newell, 1983; Yumura, 1994). The mechanisms of the chemoattraction involve the amoeba’s folate receptor, which is a G-protein coupled receptor (Wang et al., 1988; Pan et al., 2016) that elicits calcium transients, which in turn modify cytoskeletal protein dynamics (Europe-Finner and Newell, 1985; Nebl and Fisher, 1997). The identification of the signaling mechanisms that link folate/FOLR1 with regulators of the cytoskeleton and cell adhesion remodeling during neural tube formation in vertebrates demands further investigation.

FOLATE AND NEURAL FUNCTION

The involvement of folate in nervous system development and function transcends the early stages of formation of the neural tube. For instance, a dietary folic acid deficiency that results in increased homocysteine blood levels due to the deficient conversion of homocysteine into methionine, leads to a decrease in the number of proliferating neural progenitors in the dentate gyrus, affecting the neurogenesis necessary for learning and memory tasks (Kruman et al., 2005). Similarly, *in vitro* studies have shown that folate-depleted culture media results in decreased cell proliferation, differentiation and survival in the rat H19-7 hippocampal cell line (Akchiche et al., 2012). These studies argue for a mechanism whereby folate deficiency leads to an excess of homocysteine (Kruman et al., 2005; Akchiche et al., 2012), which in turn leads to aberrant homocysteinylation of neural proteins, interfering with normal neuronal development and survival (Akchiche et al., 2012).

Despite the fact that most studies have focused on the metabolism and nucleic acid synthesis-dependent mechanisms for folate action in the nervous system, other studies have found that folate also engages acute signaling in neurons. Folate induces epileptiform activity when applied in the brain of mice and rats (Spector, 1972; Baxter et al., 1973; Hill and Miller, 1974), and enhances neuronal excitability in the cat cerebral cortex (Davies and Watkins, 1973). Acute application of folate in rat hippocampal slices inhibits GABAergic transmission, enhancing excitation by blocking the postsynaptic response to GABA (Otis et

al., 1985) as well as by inhibiting presynaptic GABA receptors in the rat cuneate nucleus (Hill and Miller, 1974).

These effects on neuronal excitability argue for a specific action of folate in neural tissue and acute folate-triggered signaling independent of gene expression. Altogether these findings support a model in which folate triggers a specific signaling pathway in the neural tissues that is necessary for neural tube formation, and later may result in modulation of synaptic activity (Fig. 2).

FOLATE ACTION IN NEURODEGENERATIVE DISEASE AND NERVOUS SYSTEM REPAIR

Neural tube defects are not the only neurodevelopmental pathology associated with folate levels and function. The inherited disorder of brain-specific folate deficiency is a result of mutations in FOLR1 that manifests in late infancy and leads to developmental regression, motor dysfunction and epilepsy (Steinfeld et al., 2009; Perez-Duenas et al., 2010; Perez-Duenas et al., 2011). These FOLR1 mutants are defective in their binding to folate, and lead to inositol and choline insufficiencies, which in turn result in perturbed myelination (Steinfeld et al., 2009). This phenotype is similar to the disturbed myelination in 6-week-old rats fed with a folate-deficient diet (Hirono and Wada, 1978). Mutant forms of FOLR1 result in mislocalization of the protein and loss of FOLR1 from the cell membrane. However, the clinical severity of affected patients did not show a straightforward correlation with the altered folate binding and transport properties of FOLR1 mutants, suggesting that other factors might contribute to the clinical outcome of cerebral folate transport deficiency (Grapp et al., 2012).

Folate deficiency and an excess of homocysteine have also been associated with neurodegenerative diseases like Alzheimer's and Parkinson's disease in the adult and aging brain (Mattson and Shea, 2003; Miranda-Morales et al., 2017). High homocysteine and low folate levels in hippocampal cultures result in neuronal cell death correlated with uracil misincorporation and increased susceptibility to oxidative cell damage. Similarly, *in vivo*, mice on a folate-deficient diet exhibit increased DNA damage and hippocampal neurodegeneration (Kruman et al., 2002). Moreover, a folate-deficient diet for 3 months in uracil DNA glycosylase knockout mice leads to higher susceptibility to neurodegeneration of hippocampal pyramidal neurons, along with disturbances in neurotrophin and neurotransmitter metabolism that contribute to impaired spatial learning (Kronenberg et al., 2008).

The counterpoint of folate deficiency and neurodegeneration is evidence supporting a role for folate in the repair and regeneration of injured neural tissue (Fig. 2). Folate enhances regrowth of sensory spinal axons *in vivo* after bilateral lesion of rat dorsal root ganglia (Iskandar et al., 2004). Intraperitoneal injections with folic acid in injured rats result in enhanced axon regeneration of damaged spinal cord and optic nerves as well as functional recovery following spinal cord contusion (Iskandar et al., 2004; Miranpuri et al., 2017). The mechanism underlying folate action in axon regeneration depends on FOLR1, and is correlated with changes in DNA methylation (Iskandar et al., 2010). Matrix

metalloproteinases, such as matrix metalloproteinase-2 (MMP2), are upregulated upon spinal cord injury, and not only interfere with functional recovery but also contribute to the associated neuropathic pain (Kawasaki et al., 2008; Miranpuri et al., 2016). Folate treatment managed to reduce MMP2 expression through methylating its promoter, reducing neuropathic pain and increasing functional recovery after spinal cord contusion (Miranpuri et al., 2017).

FUTURE DIRECTIONS

Folates are pivotal molecules for the development and maintenance of all tissues. However, the nervous system seems particularly sensitive to folate action throughout development, as well as in the adult and aging brain. This argues for a potentially specific need for folate action that is different than its ubiquitous role in DNA synthesis and cell proliferation. The GPI-AP folate receptor is molecularly equipped to elicit signaling upon folate binding by partnering with transmembrane proteins and/or intracellular proteins once it has been endocytosed. Hence, the spatiotemporal profile of folate receptor expression and its interactions with distinct proteins might contribute to provide specificity to folate action in the nervous system (Fig. 2). Future studies will be needed to determine the signaling mechanisms mediated by folate and folate receptors, and the relevance of these pathways to nervous system development, maturation and disease.

Acknowledgments

We thank Dr. Andrew M. Hamilton for critical comments on this manuscript. The work in the lab has been supported by the Basil O'Connor Starter Scholar Research Award Grant 5-FY09-131 from the March of Dimes Foundation, the Klingenstein Foundation Award in Neuroscience 2008, NSF 1120796, NIH-NINDS R01NS073055 and Shriners Hospital for Children 86500-NCA, 85220-NCA and 85300-NCA grants to LNB and Shriners Hospital for Children Postdoctoral Fellowship to OAB.

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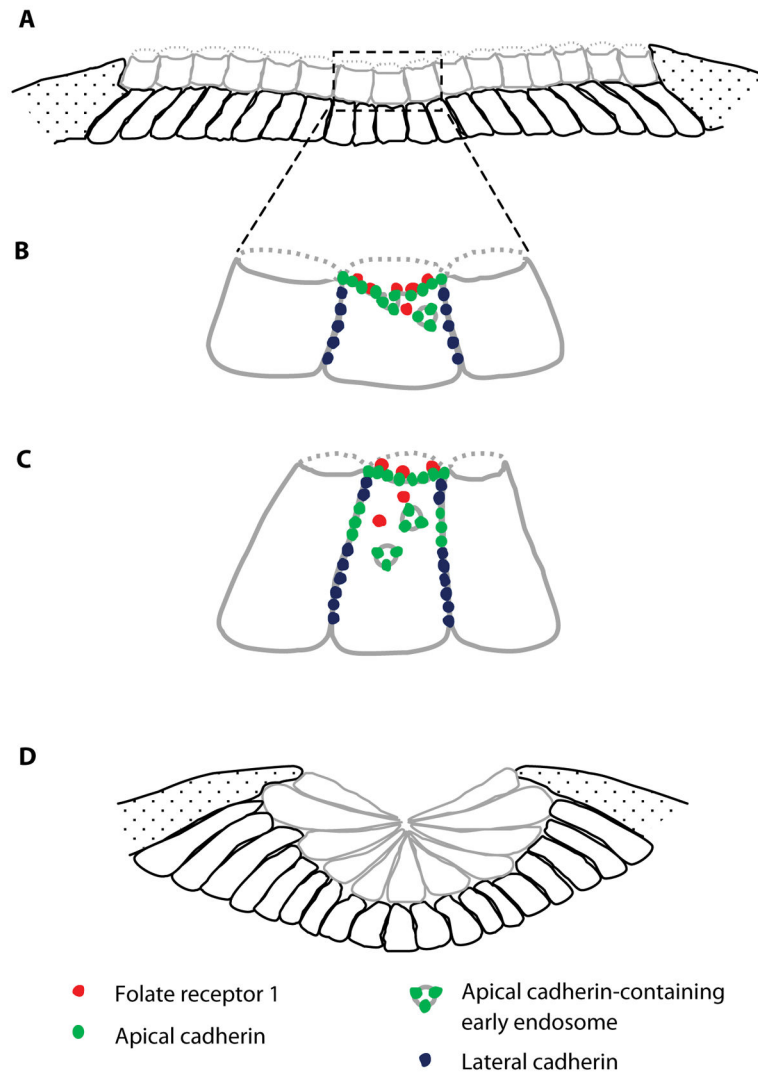
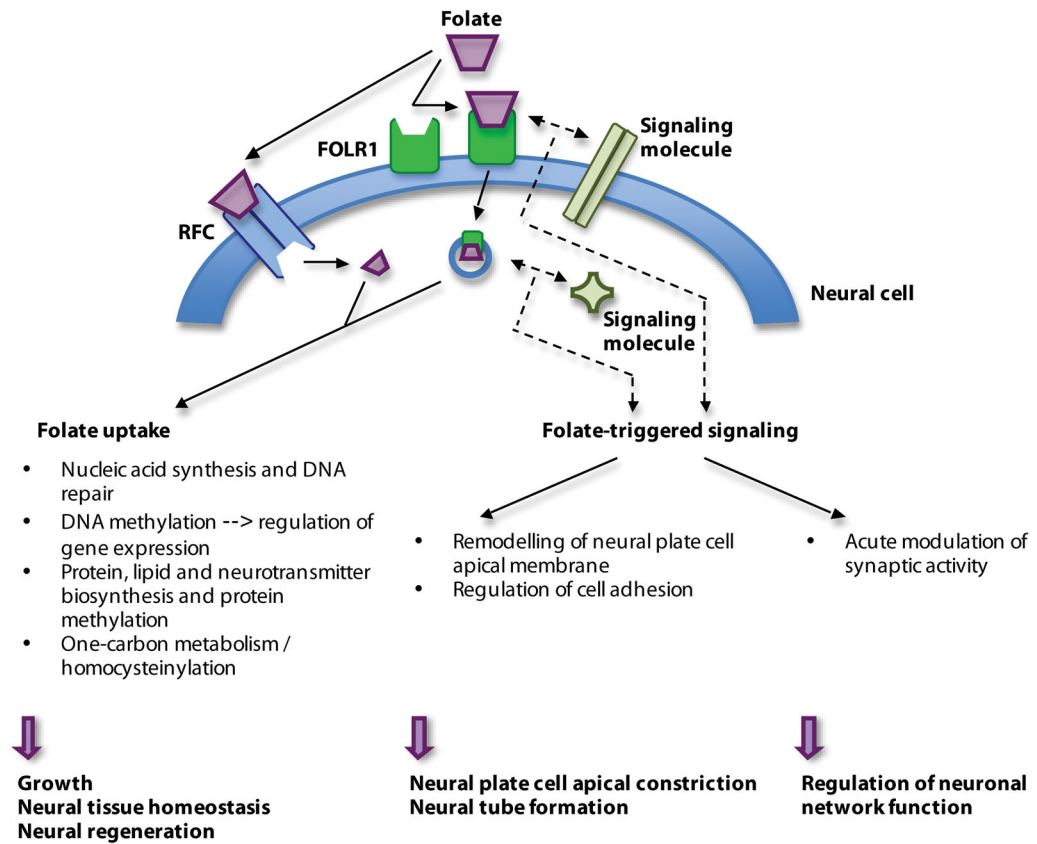


Figure 1.

Model of the role of folate receptor 1 in neural plate folding. (A) Two layers (grey – superficial, black – deep) of *Xenopus laevis* neural plate cells at the beginning of folding show a cuboidal and non-polarized morphology. Speckled regions represent non-neural ectoderm. (B) In superficial neural plate cells folate receptor 1 (red) and cadherins (green) localize to the apical membrane. Cadherins are also enriched in the cell lateral border (blue). Circles inside the cell represent early endosomes containing apical cadherin. (C) Folate receptor 1-dependent cadherin endocytosis results in redistribution of adhesion molecules and membrane from the cell apex to the lateral cell border, facilitating constriction of the apical pole and elongation of the neural plate cell. (D) Apical constriction along with other morphogenic behaviors such as cell elongation and intercalation lead to folding of the neural plate. Model derived from Balashova et al., 2017.

**Figure 2.**

Mechanisms of folate action in the developing and mature nervous system. Folate is taken into neural cells through folate receptor 1 (FOLR1) or reduced folate carrier (RFC) to be used in DNA and RNA biosynthesis, DNA repair and one-carbon metabolic reactions. These roles of folate as a vitamin are needed for neural cell division during periods of rapid growth and neural tissue repair. Alternatively, folate interacts with its receptor, triggering specific signaling mechanisms that enable the shaping of the neural plate during neural tube formation and modulation of synaptic activity to facilitate neural function.

Table 1

	PCFT	RFC1	FOLR1	FOLR2	FOLR3	FOLR4
Tissue	Intestine, liver, kidney, choroid plexus	Ubiquitously expressed	Choroid plexus, neural plate, placenta, retina, renal tubules, dorsal root ganglia neurons, dopaminergic neuronal precursors	Hemato-poietic tissues, placenta	Secreted	Oocyte
Subcellular localization	Apical brush border of small intestine epithelial cells/basal membrane of hepatocytes	Trans-membrane carrier	Apical surface neural plate cells	Unclear	NA	GPI-anchored at the oocyte cell membrane
Kinetics/mode of folate uptake	High affinity/highly efficient transporter at low pH	Low affinity/highly efficient phosphate antiporter at neutral pH	High affinity/endocytosis	High affinity/endocytosis	High affinity/endocytosis	Does not bind folate
Role	Intestinal folate absorption Folate homeostasis	Major route of folate delivery into cell of systemic tissues	Apical constriction of neural plate cells during neural tube formation	Unclear	Folate transport	Sperm-egg fusion