NEUROGENESIS
2016, VOL. 3, NO. 1, e1230167 (5 pages)
http://dx.doi.org/10.1080/23262133.2016.1230167

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COMMENTARY

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Could folic acid influence growth cone motility during the development of neural connectivity?

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ABSTRACT

Perinatal dietary supplementation, together with widespread fortification of grain-based foods with synthetic folic acid (FA) has resulted in rising concentrations of unmetabolized plasma FA in pregnant women. In a recently published study we reported on experiments in which we cultured dorsal root ganglia from chick embryos in a range of FA concentrations. We found that FA inhibited neurite extension, synaptogenesis, and growth cone motility. In this commentary we consider the possible mechanism further. The effect of FA is more likely to be on motility processes of growth cones with their exploratory filapodia than on neurotrophic stimulation. Receptors present in the filapodia membrane recognize and bind to environmental guidance cues. The presence of the NMDA receptor on filapodia, and the possible competition of FA with the neurotransmitter glutamate for binding to it, resulting in perturbation of growth cone guidance, are discussed. Whether excess FA exerts its inhibitory effects by such binding competition or via some other mechanism, further investigation is needed. Sufficient intake of folate from conception through the first month of human pregnancy is essential for neural tube closure. However, our results suggest that an upper limit for FA consumption after the first month should be considered.

ARTICLE HISTORY
Received 28 June 2016
Revised 23 August 2016
Accepted 25 August 2016

KEYWORDS
chick embryo; dorsal root ganglia; folic acid; growth cone; neurogenesis

Perinatal dietary supplementation, together with widespread fortification of grain-based foods with synthetic folic acid (FA) has resulted in a demographic with rising concentrations of unmetabolized plasma FA. The supplementation has decreased the incidence of neural tube defects in newborns,[1-5] however, there is concern that overconsumption may have adverse consequences. Evidence has begun to appear that it may affect CNS development and the risk for autism spectrum disorder.[6-9] Furthermore, some published studies have provided evidence that it does.[10-12] Yet the means by which excess FA could affect neural development has been lacking. Our recent study *Influence of Folic Acid on Neural Connectivity During Dorsal Root Ganglia Neurogenesis* published in Cells Tissues Organs[13] has provided some experimental evidence at the cellular behavior level for a mechanism by which excess FA may alter neurogenesis.

We cultured dorsal root ganglia (DRGs) taken from 8-day old chick embryos in a range of synthetic FA (pteroylmonoglutamate) concentrations. The DRGs were cultured 36 hours, then fixed and immunostained to reveal the presence of neural networks with synaptic vesicles, and then analyzed for motile behavior. We found a dose-dependent relationship with a significant reduction in the length of outgrowing neurites cultured in FA concentrations from 0.25 -20μM. The average total of stained synaptic areas surrounding each cultured DRG was significantly reduced as well. To further characterize the effects, we carried out time-lapse imaging of growth cones at terminals of extending neurites. We found that FA reduced the area-changing activity of growth cones, hindering their exploratory capabilities, along with showing a tendency to inhibit overall advancement, thus perturbing the ability to extend and form synapses. Our results showed that FA, at concentrations of 250 nM and higher reduces neurite extension and synapse formation in a dose-dependent manner during neurogenesis, and that its effect is likely mediated through inhibition of growth cone motility.[13] Although chick embryo DRG neurons are not human

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Commentary to: Wiens D, DeWitt A, Kosar M, Underriner C, Finsand M, Freese M. Influence of folic acid on neural connectivity during dorsal root ganglia neurogenesis. Cells Tissues Organs 2016; 201(5):342-353. http://dx.doi.org/10.1159/000444389

brain cortical neurons, they do form thin, straight neural processes (neurites) that elongate, led by exploratory growth cones. They do establish contact with other neurons to form neural networks. They also accumulate synaptic vesicles that will release neurotransmitters to achieve communication. These are the fundamental behaviors of all developing neurons.

Neurites lengthen with time as actin-myosin-based motility in growth cones provides a leading tractional force and microtubules and neurofilaments then assemble in the trunk of the neurite to engorge the growth cone and grow longer. This, together with our evidence that growth cone motility was depressed, suggests that the effect of FA is likely to be more directly on motility processes than on neurotrophic stimulation. Less active growth cones in the presence of high FA would predict the development of less frequent and less extensive neural networks. Growth cones are responsible for detection of favorable substrates and targets for directional guidance of innervation. If their activity is depressed, fewer contacts will be made. Similarly, differentiation to form synaptic vesicles will follow growth cone motility, becoming established where viable contacts have been made and synaptogenesis would be expected to commence. We found evidence that high FA impeded both number and extent of synaptogenic areas based on immunolocalization with a monoclonal antibody. We found support for this effect of FA on growth cones from directly analyzing motility activity using time-lapse movies. FA added to the culture environment at 2.4 μM caused a 22% reduction of growth cone area changing activity (from 19 μm^2 per minute to 15 μm^2 per minute).[13] Clearly, the growth cone's complex infrastructure of actin with associated motility proteins was strongly affected by the presence of the added FA. But in what way was it affected?

Growth cones can extend, pause, turn or retract as they navigate toward or away from a target. Their motility and guidance are achieved through dynamic assembly and remodeling of the actin microfilament and microtubule cytoskeleton that occupies them (for recent reviews see refs. 14-17). The growth cone contains a central domain occupied by stable, bundled microtubules entering from the neurite shaft, along with vesicles, organelles, and also central actin bundles. Surrounding this is a narrow transition zone where contractile actomyosin bundles form arcs perpendicular to the direction of extension and where actin filament severing for depolymerization and recycling occurs. Finally, there a peripheral domain that is filled with a fine cross-linked actin network that forms a dynamic lamellipodium, but also contains long, tight bundles of actin that extend from the transition zone outward into finger-like filapodia, supporting them and mediating their behavior. The filapodia are sensory, exploratory, and adhesive. Some microtubules, likely involved in space-filling as guidance sensors[16] extend from the central domain into the filapodia to interact with adhesion sites. Filapodia are key to directional motility, and to eventual innervation.

Receptors present in the filapodia membrane (or in membrane of the more proximal areas in the peripheral domain) recognize and bind to guidance cues present in the neurogenic environment. These may be fixed cell adhesion molecules present on other cells that are in contact with the growth cone, or they may be in the extracellular matrix environment (laminin or fibronectin, for example). In addition, there are also diffusible chemotropic molecules including neurotrophic factors, morphogens, and neurotransmitters. Binding of ligands to the receptors promotes the formation of adhesion complexes to engage the actin cytoskeleton for traction, and it initiates signaling cascades that continuously remodel the actin through polymerization, contraction, and disassembly. This involves guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), which in turn activate the Rho GTPases RhoA, Rac1 and Cdc42 locally. These then integrate and coordinate cytoskeletal effectors that control actin's behavior (see review in ref. 16).

With regard to the inhibitory effects of FA on growth cones that we reported,[13] the role of neurotransmitters and their receptors is potentially relevant. The chemical structure of FA contains the exact structure of glutamate at one end. Perhaps it could compete with glutamate for binding to a receptor. In particular, glutamate is eventually the most common excitatory neurotransmitter in the brain, and one of its receptors, the N-methyl-D-aspartate (NMDA) receptor has been implicated in synapse formation during cerebral cortex development.[17] Its presence in a developmentally regulated manner in presynaptiC-terminals has now been established in vitro and in vivo in experiments using rat neurons.[18] NMDA receptors have also been linked to developmental disorders, including epilepsy[19] and fetal alcohol spectrum disorder.[20] When

NMDA receptors in functioning synapses are activated by glutamate binding and simultaneously by glycine binding and depolarization, they open channels allowing fluxes of Na^+, K^+ and Ca^{2+}. Ca^{2+} influx is significant because it induces multiple calcium-dependent signaling networks that can alter gene expression, alter other receptors present in the postsynaptic membrane such as the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor (which also binds glutamate), and alter the local actin cytoskeleton (see review in ref. 21). Ca^{2+} influx into developing neurons via the NMDA receptor is a key regulator of neurite extension, synaptogenesis, and the maturation of synapses (reviewed in ref. 22). At a later time, such potent effects function to regulate synaptic plasticity in learning and memory, part of an activity-dependent neuronal signaling scheme.[21] Ca^{2+} influx into growth cones could certainly modulate the remodeling of the actin cytoskeleton, and therefore their "steering" as directional advance carries on. It follows that anything that could compete with glutamate's binding to the NMDA receptor, such as excess FA during development, would interfere with the process. It is of interest to note that the amino acid homocysteine also binds this receptor, and at elevated levels has teratogenic effects during development (reviewed in ref. 23).

Does FA in fact compete with glutamate for binding to the NMDA receptor? There is evidence that it can. In a voltage-clamp study of retina horizontal cell electrical current responses to glutamate binding, O'Dell et al.[24] found that FA was an effective competitor (along with several amino acids) of glutamate, blocking 40% of the response. Rowe and Ruddock[25] also showed that FA competes with glutamate to hyperpolarize retina horizontal cells of fish eyes. Our report that augmenting the glutamate concentration to 5 μM in our dorsal root ganglia cultures could overcome the inhibition of neurite extension caused by 5 μM FA[13] is consistent with this.

Whether excess FA exerts its inhibitory effects by competing with glutamate for binding or via some

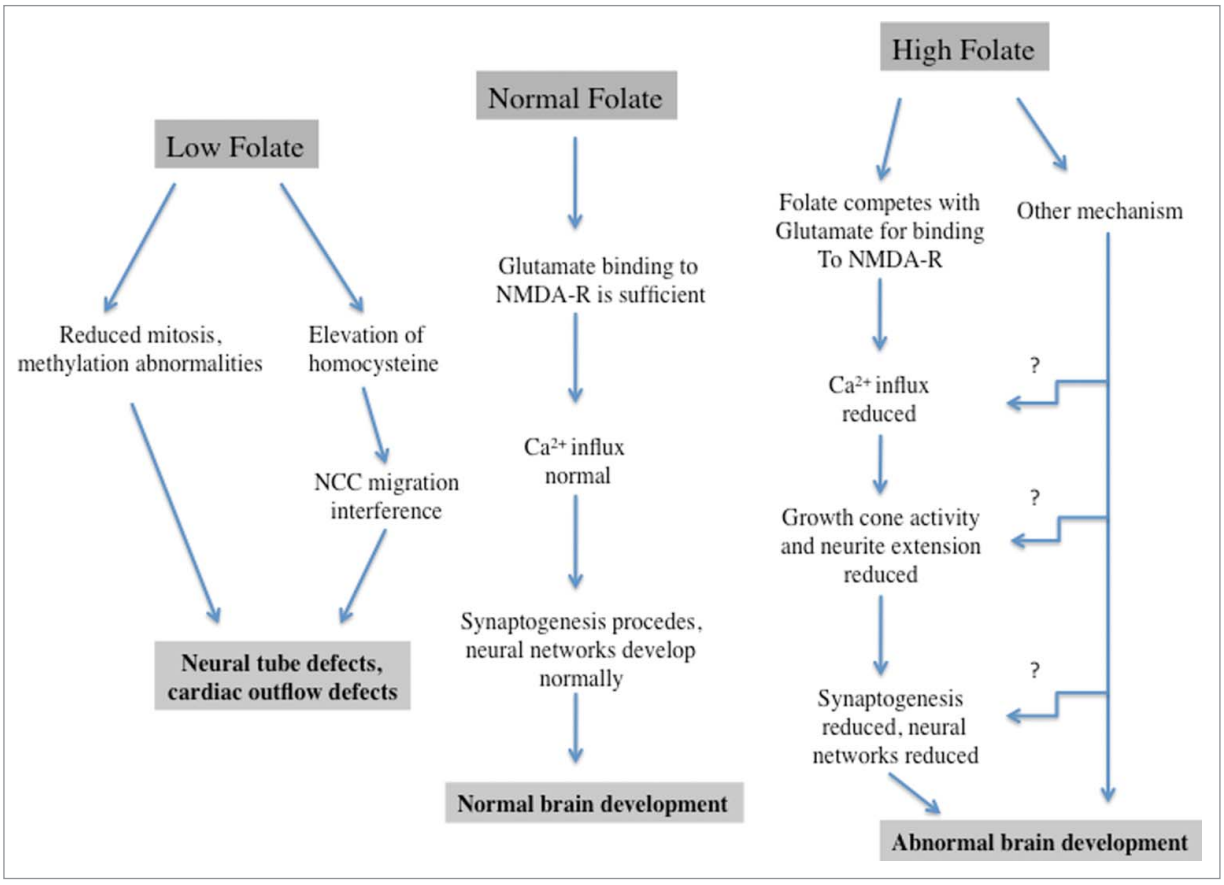

Figure 1. Scheme illustrating possible mechanisms and consequences of levels of FA that are normal, too low or too high during brain development.

other mechanism, the phenomenon should be investigated further. As we noted in our study[13] the FA concentration of fasting level plasma in adults is normally 4–45 nM (3–15 ng/mL), and in children it is 11–48 nM.[26] Adult red blood cells contain quite concentrated folate that varies widely from 317–1422 nM.[26] The concentrations of FA used in our experiment were 0.25–20 μM, and we did observe significant inhibition of neurite length at the lowest concentration, 250 nM. This is only 5–50 fold higher than in adult plasma after fasting. FA supplementation of grain-based foods and the oral supplements taken before and throughout pregnancy are elevating many women to high and continuous levels of FA consumption during the long period of embryonic and fetal brain development. Sufficient intake of folate from conception through the first month of human pregnancy is essential to make sure neural tube development will be normal, however, our results suggest that an upper limit on FA consumption after the first month should be considered. The outcomes of low, normal and high levels are illustrated in Figure 1.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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