The neural stem cell/carnitine malnutrition hypothesis: New prospects for effective reduction of autism risk?

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ABSTRACT
Autism spectrum disorders (ASDs) are developmental neuropsychiatric disorders with heterogeneous etiologies. As the incidence of these disorders is rising, such disorders represent a major human health problem with escalating social cost. While recent years witnessed advances in our understanding of the genetic basis of some dysmorphic ASDs, little progress has been made in translating the improved understanding into effective strategies for ASD management or minimization of general ASD risk. Herein, we explore the idea, described in terms of the NSC/carnitine malnutrition hypothesis, that an unappreciated risk factor for ASD is diminished capacity for carnitine-dependent long-chain fatty acid β-oxidation (FAO) in neural stem cells (NSCs) of the developing mammalian brain. The basic premise is that fetal carnitine status is a significant metabolic component in determining NSC vulnerability to derangements in their self-renewal program, and therefore to fetal ASD risk. As fetal carnitine status exhibits a genetic component that relates to de novo carnitine biosynthesis, and is sensitive to environmental and behavioral factors that affect maternal circulating carnitine levels to which the fetus is exposed, we propose reduced carnitine availability during gestation is a common risk factor that lurks beneath the genetically complex ASD horizon. One major prediction of the NSC/carnitine malnutrition hypothesis is that a significant component of ASD risk might be effectively managed from a public policy perspective by implementing a carnitine surveillance and dietary supplementation strategy for women planning pregnancies, and for women in their first trimester of pregnancy. We argue this prediction is deserving of serious clinical interrogation.
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genetic components will ultimately number in the hundreds, and genetic risk factors implicated to date include genes encoding proteins that regulate diverse cellular functions, such as synaptic development and maturation, chromatin remodeling and transcription, cytoskeleton organization, cell cycle progression, and intermediary metabolism (4, 5, 10).

Non-dysmorphic ASDs are typically diagnosed when children are two- to four-years of age because these syndromes are recognized on the basis of behavioral/social phenotypes (11). Thus, the condition has already taken root by the time it is diagnosed, and subsequent intervention necessarily falls into the ASD management category. This basic reality poses several key questions that have not been directly addressed: First, at what precise stage(s) is ASD risk first imposed? Effective prevention strategies require knowing the answer to this question. Second, are ASDs truly a collection of hundreds of diseases? If so, prevention strategies reduce themselves to what are essentially case-by-case approaches. Rapid progress under such circumstances would seem rather hopeless. But what if there is a major risk factor common to a significant fraction of the population that lurks unappreciated beneath the phenotypic horizon? That is, a common risk factor that, when combined with any one (or any other combination) of a heterogeneous cohort of other risk factors, strongly escalates the likelihood of ASD via a coincidence-reinforcement mechanism? If so, then the prospects of reducing ASD risk via a prevention regime improve dramatically once that common risk factor is identified. We are now encouraged to think this might be the case.

Herein, we explore the idea that a specific form of embryonic neural stem cell (NSC) malnutrition for the amino acid derivative carnitine forms a substantial foundation for increased ASD risk. Carnitine is a micronutrient required for the import of long chain fatty acids into mitochondria for the purpose of fueling the critical metabolic pathway of fatty acid β-oxidation (FAO) (Figure 1). FAO is not only a potent engine for ATP production, but it is a robust machine for generating reducing power and for producing acetyl-CoA—a versatile intermediary metabolite (12, 13). The idea that NSC carnitine malnutrition defines a common ASD risk factor holds the attractive feature of suggesting new strategies for mitigating ASD risk in the general population by routine prenatal carnitine supplementation during pregnancy. This strategy, should it pass clinical muster, would not only amenable to rapid deployment in the public sector, as carnitine is a natural product already in public use, but it falls under the umbrella of ASD prevention strategies rather than measures for disease maintenance.

Prenatal neocortical development

ASDs are commonly diseases of the forebrain. One region of the forebrain, the neocortex, houses the intricate circuitry responsible for higher-order functions such as perception, cognition, language, and behavior. This region of the mammalian brain is a laminated structure with distinct neuronal cell types populating distinct layers. The majority of neurons (70-80%) in the neocortex are excitatory pyramidal neurons. These cells are derived from dorsal forebrain NSCs via a prenatal developmental program (14). NSCs are unusual bipolar neuroepithelial and radial glial cells that span the entire neocortex, from the ventricular surface to the cortical plate, throughout development. During neurogenesis, NSCs proliferate rapidly to produce distinct sets of neuronal cell populations in a temporally precise manner. The first birth wave produces neurons that migrate and ultimately occupy the deepest layer of the cortical plate. Neurons born later migrate past the early born neurons and populate superficial layers of the cortical plate.

The neocortex of mouse embryos is the most commonly exploited model for studying NSC homeostasis and neurogenesis during mammalian development. NSCs of the developing mouse neocortex give birth to neurons in the window of embryonic day 11.5 (E11.5) to E17.5. While NSCs can divide to produce neurons directly or indirectly through different populations of lineage-restricted intermediate progenitor cells (IPCs), most neurons are produced indirectly through an IPC population (15, 16). These IPCs generally undergo symmetric divisions to produce two IPCs or two neurons. Before neurogenesis occurs, however, NSCs divide symmetrically to
self-expand. During neurogenesis, NSCs modify their division program to accommodate three types of divisions: symmetric self-expanding divisions to produce two NSCs; asymmetric divisions to produce one NSC and one daughter cell at more differentiated stage (i.e. either IPC or neuron); and symmetric differentiating divisions to produce two daughter cells at more differentiated stage (i.e. either IPC or neuron).

Exquisitely balanced control of NSC homeostasis is crucial for generating correct numbers and types of neurons in the developing brain (14). Inappropriate depletion of NSCs, by any one of a number of mechanisms, penalizes production of later born classes of neurons with resulting imbalances in types and numbers of neurons. Even when subtle, these imbalances are expected to enforce derangements in neuronal circuitry that result in development of a functionally compromised neocortex. In that regard, several lines of evidence implicate an NSC involvement in ASD pathogenesis by this general pathway. First, a number of ASD-risk genes encode modulators of chromatin structure that are established, or suspected, regulators of cell proliferation and/or stem cell self-renewal. Interestingly, the ever-expanding list of ASD-risk factor candidate genes overlaps significantly with analogous lists of cancer-risk genes – an overlap united by the feature that the shared genes are typically drivers of cell proliferation (17). Second, the traditional ASD-linked genes encode modulators of chromatin structure that are established, or suspected, regulators of cell proliferation and/or stem cell self-renewal. Interestingly, the ever-expanding list of ASD-risk factor candidate genes overlaps significantly with analogous lists of cancer-risk genes – an overlap united by the feature that the shared genes are typically drivers of cell proliferation (17). Second, the traditional ASD-linked genes/pathways (Wnt, PTEN, TSC1/TSC2, FMR1) also impinge on NSC self-renewal and differentiation during development (18-21), although these activities in the context of NSC biology are largely ignored in contemporary discussions of mechanisms of ASD pathogenesis. Third, magnetic resonance imaging and postmortem histological studies identify cerebral cortex anatomical abnormalities in the brains of ASD-affected patients that are consistent with deranged neuronal production from NSCs during fetal development (22, 23).

**TMLHE mutations are linked to ASD risk**
A hallmark feature of ASD is the predisposition of males to these disorders, with a male-to-female ratio approaching 4:1 (24). This sex-linked susceptibility has focused searches for X-linked mutations that show genetic associations with ASDs. It is from this perspective that we first focus on the X-linked gene *TMLHE*, and its association with ASD risk, from the perspective of genome sequencing studies. These studies form a major pillar of our hypothesis that suboptimal fatty acid β-oxidation capacity, when combined with other dietary or environmental factors, substantially elevates ASD risk for the developing fetus.

*TMLHE* encodes a trimethyl-lysine hydroxylase that catalyzes the first of four biochemical reactions involved in carnitine biosynthesis (Figure 2). Exon-focused array comparative hybridization studies identified a deletion of exon 2 of the *TMLHE* gene in a male ASD proband (25), and subsequent work demonstrated the deletion to: (i) result in severe deficiencies in TMLHE enzyme activity, and (ii) be enriched in probands from male-male multiplex ASD families relative to control males (26). Additional genetic evidence for a link between *TMLHE* deficiencies and ASD risk was obtained from X chromosome exome analyses which identified a *TMLHE* nonsense mutation (c.229C>T/p.Arg77X) that segregated with autism in one of 12 families analyzed (27). Moreover, interrogation of the *TMLHE* coding sequence from 501 unrelated male ASD probands identified two additional missense mutations (c.730G>C/p.Asp244His and c.1107G>T/p.Glu369Asp) that were absent in 303 healthy male controls. A case-control study linking rare complete gene knockouts on autosomal and X chromosomes to ASD risk independently identified a hemizygous *TMLHE* loss-of-function mutation in one of 1245 male ASD probands but not in 899 male controls (28), and a frame-shift mutation resulting in impaired TMLHE enzyme activity was identified in a male child with regressive ASD (29).

Other evidence also points to deficiencies in carnitine biosynthesis resulting in ASD-type disorders. For example, *BBOX1* catalyzes the last step of carnitine biosynthesis (Figure 2), and a young female who presents microcephaly, speech delay, growth retardation, and minor facial anomalies was found to carry a homozygous deletion of *BBOX1* (30). Straightforward interpretation of this patient is...
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complicated, however, as deletion of a second gene (FIBIN) was also detected.

**TMLHE deficiencies are high-incidence inborn errors of metabolism**

Whereas inborn errors of metabolism were previously thought to be rare disorders, with an estimated incidence for any one disorder at a frequency of 1 in 10,000 or lower (31), the prevalence of TMLHE deficiencies is far more common than that of any previously known inborn error of metabolism. For example, the TMLHE exon 2 deletion allele was detected at a frequency of 1 in ~350 in control males in one study (26), and 1 in ~900 control males in an independent analysis (27). Moreover, mining of the Exome Aggregation Consortium (ExAC) database comprised of exome sequencing data for 60,706 unrelated individuals without severe pediatric diseases (33,644 males and 27,062 females), confirmed that deleterious TMLHE point mutations and small insertion/deletions (indels) are remarkably common in the normal population (incidence of at least 1 in ~500 male individuals; ref. 32). These are truly startling numbers. Thus, TMLHE satisfies important criteria for a major ASD risk factor that is common to a significant fraction of the population yet lurks beneath the phenotypic horizon: (i) TMLHE is associated with autism-risk, (ii) the gene is X-linked, and (iii) mutations in TMLHE are remarkably frequent in the normal population. The remarkably high incidence of TMLHE deficiencies in seemingly healthy individuals can be interpreted as evidence that such deficits present a low penetrance ASD risk. However, as discussed below, we argue that penetrance considerations based on genetic data alone badly underestimate the association between carnitine availability, fatty acid β-oxidation capacity, and ASD risk.

**NSC homeostasis requires carnitine biosynthesis and long chain FAO**

Our interest in TMLHE derived not only from the high incidence of mutations circulating in the human population, but also from the fact that this X-linked gene is robustly expressed in the ventricular zone of developing mouse neocortex (33). That is, the region of embryonic brain where the somata of NSCs that fuel development of the neocortex reside (14). This raises the question of do TMLHE deficits and, by extension deficiencies in fatty acid β-oxidation, perturb development of the embryonic neocortex in vivo via disturbance of NSC activities?

To examine this question in a system that preserves the complex neurological niche in which NSCs reside, in utero electroporation technology was deployed to impose TMLHE deficits in individual NSCs in the neocortex of mouse embryos (34). The basic outline of the experimental design is illustrated in Figure 3. It should be noted that the IUE approach interrogates cell autonomous effects of TMLHE deletion in individual NSCs under conditions where non-autonomous factors provided by the neurological niche remain unperturbed. Using this experimental regime, shRNA-induced diminutions in TMLHE expression were imposed upon NSCs at an early stage of neurogenesis (E12.5). Such interventions resulted in a significant depletion of the NSC pool within 72 hours with no obvious compromise in neuronal migration or differentiation. Rescue experiments demonstrated NSC depletion was rescued by co-expression of a wild-type TMLHE but not by expression of the catalytic dead TMLHED244H (34) -- i.e. a mutant that was previously identified in a human ASD proband (26). Moreover, the NSC depletion phenotype was independently recapitulated by: (i) acute knockdown of carnitine acyltransferase (CPT1A) expression – i.e. the rate-limiting enzyme for FAO that produces the long-chain acyl carnitine which is imported into mitochondria, (ii) specific inactivation of the CPT1A with the small molecule inhibitor etomoxir, and (iii) by expression of a dominant-negative form of perilipin 1 that inhibits fatty acid mobilization from lipid droplets for subsequent FAO (33). These collective data established that interference with FAO activity at several independent points consistently results in depletion of FAO-deficient NSCs from the developing embryonic neocortex, and that this failure represents a cell-autonomous defect. That is, even a healthy neurologic niche is unable to supply sufficient exogenous carnitine to FAO-deprived NSCs to rescue self-renewal – indicating that endogenous NSC carnitine
biosynthesis is an important factor in maintaining biologically sufficient FAO activity. This result is consistent with measurements reporting that carnitine concentrations in cerebrospinal fluid are at least 10-fold lower than those in serum (35, 36).

There are a variety of mechanisms by which NSC depletion could occur, and a number of these have been experimentally excluded. FAO deficiencies do not predispose the affected NSCs to apoptosis, nor do FAO deficiencies derange other key cell biological features of NSCs such as apicobasal polarity, interkinetic nuclear migration, cleavage plane orientation, or cell cycle parameters (34). Rather, TMLHE deficits induce depletion of NSC pools by disturbing the exquisitely balanced NSC self-renewal division program designed to produce cells fated to differentiate to neurons while preserving the NSC pool (34). As described above, NSC cell division occurs primarily via an asymmetric self-renewing mode where one daughter cell is an NSC and the other a restricted lineage cell termed an intermediate progenitor cell (IPC) that is fated to differentiate into neurons (Figure 4A). The two minor modes of NSC self-division are symmetric divisions. In one case an NSC produces two NSC daughters, and in the other the mother NSC produces two IPC daughters. The former is an NSC-expanding division mode whereas the latter represents an NSC-depleting mode.

Two independent assays were developed to analyze the products of individual NSC divisions in the embryonic mouse neocortex in vivo as a function of TMLHE activity (34). Both assays are founded on the principle of using in utero gene transfer to silence TMLHE expression in individual NSCs, and to subsequently fate the two daughters produced by single cell divisions in order to identify which mode was followed in each division. Quantification of many such single divisions identified the distribution of cell division modes (Figure 4A). The first assay employed a two-step in utero electroporation scheme to identify daughter NSC pairs (Figure 4B), whereas the second assay used an in utero transfection scheme that reduced the efficiency of plasmid incorporation into NSCs but facilitated identification of isolated daughter NSC pairs for analysis (Figure 4C). Both assays consistently demonstrated that TMLHE deficits significantly increase the frequency of symmetric NSC-depleting divisions at the expense of symmetric and asymmetric NSC self-renewing divisions (Figure 4D) (34). NSC depletion was rescued by expression of wild-type TMLHE, but expression of the catalytically deficient TMLHE$^{D244H}$ was completely ineffective in that regard (Figure 4D; 34). Thus, TMLHE catalytic activity promotes NSC self-renewing cell division by inhibiting symmetric NSC-depleting divisions that produce two IPC daughters. Interestingly, this imbalance in NSC division mode induced by TMLHE deficiencies is fully carnitine remedial in both ex vivo brain hemisphere culture systems (34), and in vivo when pregnant female mice are offered dietary carnitine supplementation (our unpublished data). The latter result is particularly relevant as the pregnant mice in these experiments were maintained on a chow diet which is essentially a vegetarian low carnitine diet. Issues of diet will be addressed further below.

As would be expected given the phenotypes of human TMLHE loss-of-function mutants, BBOX1 null mice are born alive and are non-dysmorphic from the perspective that these animals exhibit anatomically normal brains with perhaps a very mild microcephaly, at best (our unpublished data). That result implies the existence of compensatory mechanisms whose activities can modulate developmental phenotype. In that regard, $\beta$-oxidation of medium chain fatty acids (MCFAs) is a biochemically redundant pathway, and mitochondrial import of MCFAs is carnitine-independent (37). Thus, medium-chain FAO activity is one such candidate for a compensatory mechanism. Indeed, we find inactivation of medium-chain FAO strongly exacerbates the self-renewal deficits of carnitine-deficient NSCs (our unpublished data). In that regard, the carrier frequencies of medium-chain FAO deficiency alleles in populations of Northern European origin are estimated to be as high as ~1 in 50 (38). That is, heterozygotes for these autosomal-recessive deficiencies are extremely common in the general population and provide yet another
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genetic factor linked to FAO-deficiency with the potential to contribute to ASD risk.

**TMLHE and NSC self-renewal**
The collective data suggest a key role for carnitine and mitochondrial FAO for NSC homeostasis in developing embryonic brain. What is the mechanism behind such regulation? FAO is a potent engine for both ATP production and generation of reducing power. Thus, IUE experiments were designed that employed fluorescent biosensor strategies for measuring ATP levels and redox status, in the cytosol or mitochondrial matrix, in unperturbed NSCs and in NSCs depleted for TMLHE or CPT1A (34). Whereas, FAO defects resulting from either diminutions in TMLHE or CPT1A activity had no effect on ATP levels in either of the two compartments, the mitochondrial matrix of FAO-deficient cells was oxidized relative to the mitochondrial matrix of FAO-proficient NSCs (34). One interpretation of those results is that FAO deficiencies derange an NSC fate switch that responds to mitochondrial redox state. In that regard, previous studies with NSCs of adult brain, and NSCs cultured ex vivo in neurospheres, suggest that FAO was important for maintaining both NSC ATP levels and mitochondrial redox state (39, 40). Given the generally detrimental role of oxidative stress in stem cell self-renewal (41) and implications that oxidative stress is associated with ASDs (42, 43), the idea that mitochondrial oxidative stress caused by FAO deficiencies induces NSC differentiation is an interesting one that merits further investigation.

That mitochondrial FAO is a potent generator for acetyl-CoA also deserves consideration (Figure 1). This intermediary metabolite is a versatile molecule that not only enters the tricarboxylic acid cycle, but is also the direct donor of acetyl groups for protein acetylation. That is, a class of post-translational modification that has wide ranging effects on protein activities, including that activities of histones that are central players in epigenetic regulation of gene expression. Our preliminary results in the murine model support a contribution by such a mechanism to maintenance of NSC homeostasis in the developing mammalian neocortex (unpublished data). In that regard, it is of interest that the switch from solitary to gregarious social behavior of several locust species is epigenetically controlled, and that carnitine is a key promoter of that behavioral switch (44). Thus, carnitine and FAO-fueled production of acetyl-CoA might power an epigenetic pathway conserved from insects to mammals for regulating behavior.

**Human ASD and carnitine deficiency**
Our studies on the contribution of FAO to NSC biology deploy a system where the physiological context of the manipulated NSCs is fully preserved, but the system comes with the caveat that individual NSCs are monitored in an essentially wild-type neurological niche. This is not the case in TMLHE mutant ASD patients, of course, where all NSCs in the tissue would be mutant. Furthermore, the studies described above were performed in mice. These issues raise the question of whether fetal carnitine and FAO deficiencies have any relevance to human ASD risk. Indeed, there is evidence to support a genuine association.

With regard to FAO, linkage of long-chain fatty acid (LCFA) β-oxidation deficits and ASD is suggested by neuro-developmental assessments of children with inborn errors of fatty acid β-oxidation -- regardless of fatty acid chain length (45). Very-long-chain acyl-CoA dehydrogenase (VLCAD) is required for β-oxidation of LCFAs in human tissues and, among the fourteen children with VLCAD deficiencies, four exhibited speech delay or language weakness, and one displayed ASD behavior. Of the two children with deficiencies in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), another enzyme required for β-oxidation of LCFAs in human tissues and, among the fourteen children with VLCAD deficiencies, four exhibited speech delay or language weakness, and one displayed ASD behavior. Of the two children with deficiencies in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), another enzyme required for β-oxidation of LCFAs in human tissues, one exhibited speech delay and the other was diagnosed with pervasive developmental delay consistent with ASD. Independent analysis of seven children identified by newborn screening as VLCAD deficient identified one as high on an autism spectrum subscale, and another was formally diagnosed with ASD (46). In patients diagnosed with LCHAD deficiencies prior to newborn screening, an increased incidence of ASDs has also been observed (47).
Carnitine deficiencies in ASD patients were first reported some two decades ago (48), and later confirmed in several independent studies that enrolled significant patient cohorts. In retrospective analyses of serum metabolites of 100 ASD children, 83% were found to present total and free carnitine levels below the reference mean (49). Moreover, levels of total carnitine and free carnitine were equal to or greater than one standard deviation below the reference mean in 36% and 27% of these ASD children, respectively (49). In a separate study comparing 30 autistic children with 30 healthy controls, low serum carnitine levels were found in 90% of the autistic children (50). A more recent report comparing 100 ASD children with 100 healthy controls documented reduced serum carnitine levels in 66% of the ASD children (51). While all of those studies involve children, whereas our mouse studies document NSC derangements in imposed in mid-gestation, the clinical data are nonetheless consistent with the carnitine deficiency hypothesis. In that regard, multiple studies document significant decreases in maternal serum carnitine levels during pregnancy that approach pathologically low levels (52-59). Interestingly, that decline in carnitine levels commences at the onset of mid-gestation (12 weeks; 58,59), a window that corresponds to the one where we interfere with carnitine synthesis and fatty acid β-oxidation in our mouse in utero electroporation experiments (34). While the functional significance of those associations with regard to ASD risk has not been interrogated, those data do suggest that maternal circulatory carnitine is often an unreliable source of this micronutrient to the developing fetal brain.

**TMLHE mutations and the phenotypic penetrance paradox**

Although TMLHE is an ASD-linked gene, contemporary thought holds that the linkage is weak because the penetrance of TMLHE mutations is poor. The argument to this effect comes from genome sequencing data that, while identifying TMLHE mutations as high-incidence inborn errors of metabolism, also indicate TMLHE-deficient humans do not typically present ASD symptoms (26, 27). Based on the frequency of the TMLHE exon 2 deletion in male-male multiplex ASD families, the phenotypic penetrance of TMLHE mutations is estimated to be an unimpressive ~3% (26). However, genomics-based arguments assume genetic status will accurately translate to circulating carnitine status. In our view, this base presumption is unjustified as it ignores common behavioral and environmental factors that will independently influence maternal carnitine status. That is, the major carnitine supply available to the fetus, and the sole carnitine resource for the TMLHE-deficient fetus. We suggest maternal behavior and environment play far larger roles in the regulation of NSC carnitine status in the developing human fetus than currently appreciated, and that these non-genetic influences confound direct extrapolation of the genomics data.

The first argument to this effect comes from examination of the biochemistry underlying carnitine biosynthesis. The first and the last enzymatic reactions in the conversion of trimethyl-lysine to carnitine are catalyzed by the *TMLHE* and *BBOX1* gene products, respectively. Both enzymes are Fe-dependent dioxygenases. Carnitine biosynthesis is therefore sensitive to iron homeostasis. Clinical evidence to support this contention derives from studies where reduced serum carnitine levels are routinely detected in children suffering from iron-deficiency anemias (60,61). Regarding NSC carnitine malnutrition, fetal iron-deficiency follows maternal iron-deficiency, and this syndrome is common. Iron-deficiencies afflict 30-50% of pregnant women in the United States with higher incidence globally (61-65). Thus, this extremely common non-genetic condition potentially represents a far more significant pathway for reducing fetal NSC carnitine levels than do the TMLHE mutations themselves. Indeed, epidemiological studies report iron deficiency is associated with substantially elevated autism risk (66,67).

Behavioral factors also impact maternal, and therefore fetal, carnitine status. Although humans have the capacity to synthesize carnitine de novo from trimethyl-lysine, that capacity is limited. The primary reservoir of carnitine comes from the diet where some 75% of the circulating carnitines in the healthy human body
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are derived from exogenous sources (68,69). Whereas the trimethyl-lysine that fuels carnitine biosynthesis is rich in plants, vegetables, fruits, fish and dairy products represent carnitine-poor dietary sources. Red meat, however, is a particularly rich carnitine source. For purpose of comparison, normalizing the data per unit mass where carnitine equivalents in beef are set at 1.00, pork (0.3), fish (0.06), chicken (0.04) and milk (0.10) are inferior carnitine sources (68). Plants are much poorer sources. Mass spectrometry-based measurements indicate plant carnitine levels are several orders of magnitude lower than those present in animals (70). Thus, maternal diet during pregnancy is a potentially significant behavioral variable that impacts fetal NSC carnitine supply. As vegetarianism and veganism are ever more popular dietary practices, these behavioral factors also potentially represent frequent pathways for reducing fetal NSC carnitine levels. Indeed, clinical data report that vegetarian and vegan women present significantly lower plasma carnitine levels than do mixed-diet women (69,71,72).

In situ synthesis of carnitine is limited to several tissues in mammals (liver, kidney, brain and perhaps intestine; refs. 68,69,73-75). Renal re-absorption plays a particularly critical role in systemic carnitine homeostasis as circulatory carnitines are vigorously salvaged by the kidney. This salvage activity is mediated via the high-affinity carnitine transporter OCTN2 encoded by the SLC22A5 gene (69,73). OCTN2 sets the renal saturation threshold for carnitine excretion at ~50 \( \mu \)M – a value that closely coincides with normal plasma carnitine concentrations (25-50 \( \mu \)M). Thus, renal carnitine reabsorption is highly efficient when circulatory carnitine concentrations are low. Any pathogenic conditions during pregnancy (e.g. infection), or pharmacological treatments (e.g. valproate; 76), that significantly impair either carnitine salvage or transport, could expose fetal NSCs to elevated risk of reduced maternal carnitine supply. The consequence would be suboptimal FAO activity during a critical developmental window. Moreover, environmental intoxication with heavy metals such as zinc, lead and cadmium is also associated with impaired Fe-homeostasis (77). Exposure of the pregnant female to high levels of heavy metals in drinking water, or otherwise, also carries the risk of impairing de novo carnitine biosynthesis.

We emphasize that genetic and environmental factors independent of carnitine status, but associated with mitochondrial FAO activity, are also relevant. Carnitine-independent \( \beta \)-oxidation of MCFAs provides an example. One newborn screening study reported that thirteen of twenty-seven children identified as suffering medium-chain FAO deficits presented language/speech delay or frank speech deficits, one child exhibited autistic behavior, and four other children exhibited developmental deficits related to motor function (45).

The NSC-carnitine malnutrition hypothesis: Implications and questions

The principal thesis of this article is that NSC carnitine malnutrition poses a major unappreciated risk factor for ASD. The foundations for this argument include the fact that the X-linked TMLHE mutations are associated with ASD risk and show a remarkably high incidence in the population. The risk for NSC malnutrition is further compounded by environmental and behavioral factors that impinge on NSC carnitine supply independently of genetic status. These include maternal iron deficiencies, kidney dysfunction, heavy metal intoxication, and lifestyle factors such as maternal diet. The experimental foundation for the NSC/carnitine malnutrition hypothesis comes from our study of the consequences of carnitine and FAO deficiencies in murine embryonic NSCs during neurogenesis (34). That murine NSC study raises interesting ideas and questions as these relate to ASD risk. Four ideas deserve particular emphasis.

First, the detrimental effects of carnitine and FAO insufficiencies on NSC self-renewal are apparent in mice by E15.5. That is, at a developmental stage that translates to mid-gestation in humans. This conclusion projects that prevention strategies for ASDs of either this specific etiology, or those to which carnitine-deficiencies contribute when combined with some other risk factor(s), will not be effective if these rely on behavioral diagnoses in young children. That timing of diagnosis and intervention is well behind the curve in terms of
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deploying any meaningful prevention strategy. Our mouse studies indicate the seeds for NSC dysregulation (and inferentially ASD risk) are already sown in mid-gestation (34). In this regard, we find the well-documented decline in carnitine levels that commences at the onset of mid-gestation in pregnant women to be of particular interest (52-59).

Second, the data speak to limitations in sole reliance on prenatal genetic testing for TMLHE mutations in devising strategies for ASD prevention. The environmental and behavioral factors that can give rise to carnitine insufficiencies likely far outstrip genetic factors in terms of significance. Routine monitoring of circulating carnitines in pregnant women via direct biochemical measurement describes a superior solution. Prenatal genetic testing remains beneficial in alerting those cases where a parent is recognized as carrier of a TMLHE mutation, or the embryo is recognized as genetically deficient in de novo carnitine synthesis. In those cases, targeted monitoring of circulating maternal carnitines throughout pregnancy might be of value as it is the FAO-deficient fetus that will be particularly vulnerable to diminutions in those metabolites.

Third, consideration of maternal diet as a behavioral factor of relevance to ASD risk has its own interesting implications. This hypothesis raises the question of whether adoption of vegetarian or more extreme vegan lifestyles by women of child-bearing age, especially by pregnant women, inadvertently raises the specter of ASD risk for the developing fetus. The biochemical rationale for this idea is that women embracing such lifestyles would be predisposed to reductions in circulating carnitine concentrations (69,71,72). This idea has demographic implications. It is an interesting conjecture that appropriately designed cross-correlation analyses will identify such behaviors (and therefore rising ASD incidence) to be concentrated in affluent, and most likely socially progressive, urban communities. There is some evidence to support this idea (78).

Fourth, with regard to demographics, the genomics studies that identified TMLHE mutations as frequently occurring inborn errors of metabolism were weighted heavily in favor of populations of European descent (26, 27). One extrapolation of those data is that TMLHE mutations were tolerated, and therefore fixed, in that population because red meat has represented a dietary staple in that part of the world for thousands of years. That is, genetic deficiencies in carnitine synthesis were tolerated in the European population because their effects were nutritionally complemented by a carnitine-rich diet. In this manner, the selection pressures that would have otherwise eliminated such mutations from the population were effectively removed.

What is the incidence of TMLHE mutations in other populations/races where red meat has not been such a central dietary staple over the course of human history? The selection pressures for appropriate FAO activity would have remained intact in those populations over the course of their history -- with the result that TMLHE mutations would be strongly disadvantaged in those cohorts. Interrogation of this question is within reach of existing genome sequencing technology, and we are of the opinion that there is now a significant rationale for examining this issue in detail.

Implementable prospects for reducing ASD risk?

When the non-genetic factors that impinge on carnitine homeostasis in the mother and fetus are considered in aggregate, we estimate that some 20-30% of pregnant women in the United States might be exposing the developing fetus to a suboptimal carnitine environment (the frequency of iron-deficiency alone meets this level; 61-65). Even an ASD penetrance of 3-5% in such potentially at-risk pregnancies would account for a very significant fraction of ASD cases. Thus, the NSC/carnitine malnutrition hypothesis calls for clinical exploration of what would be a direct and rapidly implementable strategy for mitigating a potentially major and unappreciated ASD risk factor. That is, designation of carnitine as a recommended daily supplement for pregnant women. Our finding that NSC self-renewal imbalances caused by carnitine/FAO deficiencies in mice are remediated by supplementation of the maternal diet with carnitine supports the case (34, our unpublished data). As carnitine is a natural product, one already available to the public over the counter, clinically-approved implementation of this...
strategy would not require the expensive and
time-consuming process of securing approval by
the Food & Drug Administration. Obviously,
daily recommended intakes need to be clinically
determined as carnitine is a precursor to
trimethylamine N-oxide (which has been
implicated in cardiovascular disease; ref. 79),
and high carnitine doses can also have other
undesirable consequences (29,80). However,
clinical management of safe dosage for pregnant
women does not seem to pose a particularly
complicated hurdle. Carnitine use in adults for
the purposes of improving brain function, blood
pressure, and heart performance already
indicates daily intake of 2g carnitine is safe (81).
Indeed, a daily 500mg carnitine dose is
sufficient to correct the carnitine decline
experienced during pregnancy with no reported
ill effects to mother or to fetus (59). Moreover,
with regard to fetal health, carnitine
supplementation of parenterally fed babies born
prematurely (<37 weeks old) has been practiced
for decades with no obvious ill effects (82) –
suggesting that carnitine supplementation during
pregnancy is very likely to be well-tolerated by
the fetus.

What is there to lose from the public
health perspective by investing serious clinical
effort in investigating a broad-based carnitine
supplementation strategy? Essentially nothing,
and the data suggest there is much to gain. A
clinically-approved carnitine supplementation
program would be analogous to the position
taken in 1992 by Public Health Service to
recommend a daily dose of 400µg folate for all
women of childbearing age as a mechanism to
mitigate neural tube birth defects. This
recommendation was subsequently extended by
a Food & Drug Administration decision in 1996
to require folate enrichments be added to a
variety of grain products. Implementation of
folate supplementation represents arguably the
most successful public health policy enacted to
date. Perhaps a carnitine supplementation
program will prove similarly successful.

Carnitine and cognitive development in early
colorhood
Although the focus of our work thus far has been
on the consequences of carnitine/FAO
deficiencies on embryonic NSC biology (34),
and we posit that it is at the fetal stage where
carnitine supplementation will first be effective
as prevention strategy, there is evidence to
suggest that carnitine also plays a role in post-
natal brain development in young children. One
case in point involves a male child who carries a
TMLHE mutation, and who was diagnosed with
ASD at four years of age (29). A daily oral
carnitine delivery regimen resulted in significant
improvements in several key behavioral
milestones. Due to secondary gastric effects
caused by the high carnitine doses delivered, the
supplementation dose for this patient was
cycled. Importantly, cognitive improvements
cycled in phase with carnitine supplementation
(29). This correlation lends confidence that the
beneficial effects were directly related to
carnitine supplementation, and is suggestive of
some post-natal benefits. A follow-up pilot
study also provides supporting, albeit
preliminary, data (80).

Also with regard to potential postnatal
benefits of carnitine supplementation, we note
that the NSC/carnitine malnutrition hypothesis
does not adequately account for the sex
disequilibrium signature of ASDs. It would
seem that environmental factors that result in
fetal carnitine deficiencies would predominate
over X-linked genetic factors such as
TMLHE mutations. While sex-linked contributions
emanating from hormonal differences might
help account for the signature male bias in
ASDs, an interesting idea has been proposed by
Beaudet that young males might be intrinsically
reduced in the capacity to transport carnitine
across the blood-brain barrier (83). This sex-
linked deficit is suggested to be due to reduced
expression of an amino-acid transporter
expressed from an X-linked gene
SLC6A14 that
might escape X-inactivation (83).

In that regard, the diets of newborns and
young children during their first 18 months of
life are typically low in carnitine content.
Nursing is followed by introduction to solid
food via fruit and cereal-dominated diets.
Perhaps simple adjustments, such as offering
toddlers a small daily dose of meat broth, will
deliver as yet under-appreciated benefits in
potentiating cognitive development –
particularly for children with in-born errors in
carnitine biosynthesis and fatty acid β-oxidation.
There is a growing body of evidence to suggest such measures have merit (83, 84).

**Concluding thoughts**

Finally, we are struck by the fact that two developments dominating public interest in contemporary news cycles detail the seemingly unrelated topics of: (i) the alarming rise of autism in young children, and (ii) the damaging human health and planetary-scale environmental costs associated with cattle farming and consumption of red meat (85). The meteoric rise of companies promoting adoption of meatless mimics for beef and chicken at major fast-food outlets testifies to the rapidly growing societal appetite for reducing meat consumption. This philosophy is even rising to the level of circulation of scientific petitions exhorting world governments to unite in adopting global measures to restrict meat consumption (86). We now pose the question whether such emerging societal attitudes regarding nutrition, and its environmental impact, are on collision course with increased ASD risk. Food for thought, indeed.

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**CONFLICT OF INTEREST**

The authors declare no financial conflicts.

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FIGURE LEGENDS

Figure 1. Carnitine-dependent long-chain fatty acid β-oxidation produces ATP, reducing power and acetyl-CoA. Carnitine-activated long-chain fatty acids (long-chain acyl-CoA) are transported into the mitochondrial matrix for β-oxidation. This import reaction is initially catalyzed by the carnitine acyltransferase CPTI followed by the actual transport step and finally be reconversion of the acyl-carnitine to acyl-CoA (not shown). Within the mitochondrial matrix, long-chain acyl-CoA undergoes multiple cycles of β-oxidation to generate acetyl-CoA, which then enters tricarboxylic acid cycle (TCA) or serves as donor of acetyl groups for prost-translational modification of proteins. Both fatty acid β-oxidation (FAO) and TCA cycle activities generate the reduced coenzymes NADH and FADH2, which are consumed in ATP production via the electron transport chain (ETC). By supplying acetyl-CoA to the TCA cycle, β-oxidation increases generation of the TCA intermediates isocitrate and malate in the mitochondria and cytoplasm. Isocitrate and malate are subsequently used as substrates by isocitrate dehydrogenases (IDHs) and malic enzymes (MEs), respectively, to generate reducing power in the form of NADPH both in the mitochondria and in the cytoplasm.

Figure 2. Carnitine homeostasis in brain. Brain cells synthesize carnitine de novo and also import exogenous carnitine from interstitial cell fluid. Under normal conditions, levels of circulating carnitine are primarily set by dietary carnitine intake and renal carnitine reabsorption. De novo biosynthesis of carnitine is limited in mammals. The process initiates with TML (trimethyl-lysine) and involves four biochemical reactions sequentially catalyzed by TMLHE (trimethyl-lysine hydrolase, epsilon), HTMLA (hydroxyl trimethyl-lysine aldolase), TMABADH (trimethylaminobutyraldehyde dehydrogenase), and BBOX1 (γ-butyrobetaine hydroxylase 1), respectively.

Figure 3. In utero electroporation as an approach to interrogate TMLHE function in NSCs. In a survival surgery procedure performed on a pregnant mouse, a mixture of plasmids (for expressing EGFP and either control or Tmlhe shRNA) is injected into the lateral ventricle of the mouse embryo. Short electric pulses are delivered across the head of the embryo to induce uptake of the plasmids by cells with access to the lateral ventricle (predominantly NSCs, which extend a basal process to the pial surface and an apical process to the ventricular surface). Electroporated embryos are returned to the uterus and allowed to incubate in utero for an appropriate period dictated by experiment (72 hours in this case), during which transfected NSCs divide to self-renew and to produce differentiated cells (IPCs and neurons). Embryos are subsequently sacrificed, and confocal imaging analyses of fixed and immune-stained brain sections are executed to fate the EGFP+ cells as either NSCs or IPCs (numbers indicated in the figure; see ref. 33). The fractional representation of NSCs was significantly reduced in the Tmlhe knockdown group compared to control shRNA group. In this assay, only cell-autonomous effects are monitored as the transfected NSCs are surrounded by non-transfected NSCs and therefore reside in an unperturbed neurological niche. Interference with carnitine synthesis, with mobilization of fatty acids from lipid droplet stores, or with import of acyl-carnitines from the cytoplasm into the mitochondria consistently results in depletion of NSCs from the ventricular zone of the developing neocortex (33).

Figure 4. TMLHE-deficiencies impair the NSC self-renewal program. (A) Modes of NSC divisions. NSCs generate neurons primarily through TBR2+ IPCs. Only this route of neurogenesis is illustrated in the figure. Bipolar and multipolar cells in the figure represent NSCs and TBR2+ IPCs, respectively. During neurogenesis, NSCs undergo three types of divisions: symmetric self-renewing divisions to generate two NSCs; asymmetric divisions to generate one NSC and one IPC; and symmetric differentiating divisions to generate two IPCs. (B) Two-step in utero electroporation assay for analyzing individual NSC divisions (33). EGFP mCherry+ cell pairs are well isolated from each other and therefore can be confidently...
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identified as two daughter cells derived from the division of a common mother NSC. (C) Attractene transfection strategy for analyzing individual NSC divisions (33). Co-injection of the plasmid solution with Attractene reagent without electroporation leads to low transfection efficiency of NSCs lining the lateral ventricles. Thus, from a single transfected NSC (cell fate analyzed 24h after DNA/Attractene injection), the adjacent pair of EGFP+ daughter cells from the same initially transfected NSC can be confidently identified. (D) Results obtained from the two experimental regimes were similar (33). In both assays, the vast majority of NSC divisions in control groups (control shRNA, or Tmlhe shRNA co-expressed with shRNA-resistant wild-type TMLHE) were self-renewing with at least one daughter cell as NSC. Under conditions of TMLHE-deficiency (Tmlhe shRNA with or without co-expression of the TMLHE^D244H mutant), nearly half of the divisions exhibited by TMLHE-deficient NSCs were symmetric differentiating divisions that lead to stem cell depletion.
Figure 1

Acetylation of mitochondrial, cytoplasmic, and nuclear proteins

Long-chain acyl-CoA
Carnitine

CPTI
Long-chain Acyl-CoA

NADPH
Pyruvate
Malate
NADH/FADH2

Cytosol

Mitochondrial matrix

FAO
Acetyl-CoA
TCA

ATP

NADH/FADH2

ME1

IDH1

IDH2

α-ketoglutarate

oxaloacetate

Isocitrate

Citrate

EtC
Figure 2

Dietary carnitine
Renal reabsorption

Blood brain barrier

Circulating carnitine

Brain Cell

Carnitine

TML
TMLHE
HTML
HTMLA
TMABA
TMABADH
γ-BB
BBOX1
Figure 3

Plasmids (EGFP+Tmlhe shRNA) injected into lateral ventricle

Non-transfected NSCs

E12.5

Ventricular zone

Transfected NSCs

Control shRNA

Tmlhe shRNA

E15.5

% of EGFP+ cells that were NSCs: 17.37 ±2.24 (mean±SD)

E15.5

% of EGFP+ cells that were NSCs: 5.1 ±1.6 (mean±SD)
Figure 4

A. Embryonic neocortex

Symmetric self-renewing  |  Asymmetric  |  Symmetric differentiating

Lateral ventricle

B. Electroporate with EGFP + control or Tmlhe shRNA

Electroporate with mCherry plasmid 30h later

Analyze identity of EGFP/mCherry* cell pairs 20h later

C. Plasmids + Attractene injected into lateral ventricle

Transfected NSC 24h later

D. 2-step IUE: EGFP/mCherry* cell pairs

Self-renewing 81% Symmetric differentiating Tmlhe shRNA 29% Symmetric differentiating 41%

Attractene transfection: EGFP* cell pairs

Self-renewing 59-65% Symmetric differentiating 5-12% Control shRNA TMLHE rescue

Self-renewing 40-45% Symmetric differentiating 41-54% Tmlhe shRNA TMLHE^{D26H} rescue
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