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Mitochondrial Energy-Deficient Endophenotype in Autism

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Abstract: While evidence points to a multigenic etiology of most autism, the pathophysiology of the disorder has yet to be defined and the underlying genes and biochemical pathways they subserve remain unknown. Autism is considered to be influenced by a combination of various genetic, environmental and immunological factors; more recently, evidence has suggested that increased vulnerability to oxidative stress may be involved in the etiology of this multifactorial disorder. Furthermore, recent studies have pointed to a subset of autism associated with the biochemical endophenotype of mitochondrial energy deficiency, identified as a subtle impairment in fat and carbohydrate oxidation. This phenotype is similar, but more subtle than those seen in classic mitochondrial defects. In some cases the beginnings of the genetic underpinnings of these mitochondrial defects are emerging, such as mild mitochondrial dysfunction and secondary carnitine deficiency observed in the subset of autistic patients with an inverted duplication of chromosome 15q11-q13. In addition, rare cases of familial autism associated with sudden infant death syndrome (SIDS) or associated with abnormalities in cellular calcium homeostasis, such as malignant hyperthermia or cardiac arrhythmia, are beginning to emerge. Such special cases suggest that the pathophysiology of autism may comprise pathways that are directly or indirectly involved in mitochondrial energy production and to further probe this connection three new avenues seem worthy of exploration: 1) metabolomic clinical studies provoking controlled aerobic exercise stress to expand the biochemical phenotype, 2) high-throughput expression arrays to directly survey activity of the genes underlying these biochemical pathways and 3) model systems, either based upon neuronal stem cells or model genetic organisms, to discover novel genetic and environmental inputs into these pathways.

Key words: Carnitine, oxidative stress, 15q, SIDS, calcium, valproate, serotonin

INTRODUCTION

The autism spectrum disorders (ASD) are a group of behaviorally defined developmental disorders that all share the same characteristic core deficits in social interaction, communication and behavior, however, the pathophysiology of these disorders has not yet been defined [1, 2]. Evidence suggests a multigenic etiology of many, if not most cases of ASD; however the underlying disease-susceptibility genes and the biochemical pathways they subserve remain unknown [2, 3, 4]. Identifying such alterations in autistic individuals provides an added dimension to their phenotyping, refining within this heterogeneous spectrum a more coherent subgroup (an endophenotype) to which they belong. It additionally provides an advantage because the genes involved in the pathway become implicated as potential disease candidate genes involved in the etiology of ASD, a hypothesis that can be readily tested by association studies in a well-defined patient cohort [5, 6, 7]. Those genes additionally potentially serve as new molecular diagnostics of the disease and as targets for the development of new classes of medications.

Mitochondrial energetics, oxidative stress and autism: The high heritability of ASD assures us that genes and the biochemical pathways they subserve, underlie the phenotype. To date these pathways remain

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elusive, but there are now several suggestions in the literature that oxidative stress [8, 9, 10] and mitochondrial dysfunction are abnormal, perhaps, in turn, impacting a variety of downstream processes. Oxidative Stress (OS) describes the steady state level of oxidative damage in a cell, tissue, or organ, caused by the reactive oxygen species (ROS). ROS, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms [11]. Most reactive oxygen species come from the endogenous sources as by-products of normal and essential metabolic reactions, with energy generation from mitochondria accounting for the vast majority, but also including essential metabolic processes such as peroxisomal oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, pathogen-stimulated neutrophil phagocytosis, arginine metabolism and a host of tissue specific enzymes. The level of oxidative stress is determined by the balance between the rate at which oxidative damage is induced and the rate at which it is efficiently repaired and removed.

Recent studies have described findings of increased oxidative stress in children with autism compared to age- and gender-matched normal controls [12, 13]. The oxidative stress in autism is suggested to be caused by the imbalance between the generation of ROS by endogenous factors (e.g. nitric oxide, xanthine oxidase and homocysteine) and/or exogenous (environmental) factors e.g. (air pollutants, valproic acid and viral infection) and the defense mechanism against ROS by antioxidants [14].

Mutations in mitochondrial DNA cause disease: The first inherited mitochondrial DNA (mtDNA) disease mutation was identified by Wallace and coworkers, a missense mutation at nucleotide pair (np) 11778 in the mitochondrial respiratory complex I gene, *ND4*, causing Leber's Hereditary Optic Neuropathy (LHON) [15]. It helped establish the paradigm for all mitochondrial diseases that create a wide range of apparently different tissue-selective energy deficiency syndromes [16]. The diseases transmitted by mtDNA all show a *maternal inheritance* pattern, since all mitochondria are transmitted via the egg, virtually none via the sperm. This means that all offspring of a transmitting female inherit *some* of the mutant mitochondria. Since the egg contains several hundred mitochondria, each with several copies of the small circular mtDNA that encompasses the full mitochondrial genome, generally there is a mixed population of normal and mutant mitochondria transmitted, a feature termed *heteroplasmy*. LHON was an opportune exception since this mutation, being relatively weak and nonlethal, is generally found to be a *homo*plasmic cause of mid-life blindness. The relatively uniform presentation of the LHON syndrome and the homoplasmic mtDNA made this a good prototype in which to recognize a mutant mtDNA inheritance pattern. For most mitochondrial diseases this is not so obvious because of the variability produced by heteroplasmy. Even within a family one commonly observes a very wide range of presentations of apparently different diseases that all ultimately prove to be caused by a given inherited mutation. These syndromes reflect varying degrees of heteroplasmy and differing *threshold* effects of the mutation in different energy-intensive tissues. Characteristic presentations thus include mitochondrial encephalomyopathy, cardiomyopathy and a range of endocrine, hepatic or renal tubular dysfunctions. For example, mtDNA missense mutation at np 8993 in the respiratory complex V gene *ATP6* is a strong, lethal allele that is therefore invariably heteroplasmic and found to be associated with a wide range of clinical presentations. When patients inherit a mix of mitochondria with about 75% of the mtDNA carrying the mutant allele, they develop retinitus pigmentosa; at 85% mutant they develop macular degeneration, mental retardation and olivopontocerebellar atrophy; and at 95% mutant, they develop the early lethal metabolic lactic acidopathy of Leigh's Syndrome.

Since the mitochondria contain all of the machinery needed for their own protein synthesis, many pathological mutations affect rRNA and tRNA genes, affecting all proteins encoded by the mtDNA and causing global mitochondrial dysfunction. The first of these protein synthesis mutations was discovered by the Wallace lab in the $tRNA^{Lys}$ gene at np 8344 [17]. This mutation causes complex pediatric and adult neuromuscular disease with symptoms varying markedly depending on the percentage of heteroplasmy. The most characteristic symptoms are myoclonic epilepsy and mitochondrial myopathy with ragged red muscle fibers (MERRF), but patients can present with hearing loss, dementia, cardiomyopathy or even the lethal childhood Leigh's Syndrome. Another common mtDNA protein synthesis mutation occurs at np 3243 in the $tRNA^{Leu(UUR)}$ gene. At a high percentage mutant (75-95%), this mutation can cause mitochondrial myopathy, stroke-like episodes and/or cardiac conduction defects and cardiomyopathy. However, at low levels of mutant (10-30%), this same mutation causes what appears to be a very different disease, Type II diabetes [18]. In fact, it now appears that about 1% of

all Type II diabetes, *a classic heterogeneous complex polygenic disease like autism*, is due to this one mtDNA mutation, making it by far the most common known cause of diabetes mellitus. Many other mtDNA mutations that affect mitochondrial protein synthesis can also be expressed as type II diabetes and it is known that a patient is four times more likely to inherit type II diabetes from his mother than his father, consistent with mtDNA's pattern of inheritance [18]. Finally, mild mtDNA mutations have been associated with late-onset diseases [16]. For example, mtDNA $tRNA^{Gln}$ mutation at np 4336 accounts for about 5-7% of late-onset Alzheimer's Disease (AD) and Parkinson's Disease (PD) [19], again examples of *complex polygenic diseases like autism*. This is consistent with repeated reports that AD and PD are associated with defects in mitochondrial respiratory complexes IV and I, respectively. Many additional pathogenic mtDNA mutations are listed at www.mitomap.org [20]. Hence, there is already ample evidence that mtDNA mutations can cause pediatric and adult neurological and metabolic disease and can produce disease phenotypes recognized to be characteristically-transmitted as complex polygenic traits, such as autism.

Mutations in nuclear genes encoding mitochondrial components cause disease: A large, but still incompletely ascertained number of hundreds of nuclear DNA- encoded (nDNA) proteins also contribute to mitochondrial function, ranging from component proteins of the respiratory complexes themselves to those involved in its regulation or assembly and these nDNA encoded mitochondrial genes are dispersed throughout the chromosomes[21]. For example, simply observing the widely distributed chromosomal locations of the 36 mitochondrial respiratory complex I nDNA genes that encode just the flavoproteins, iron-sulfur proteins and array of hydrophobic proteins composing this multi-protein enzyme assembly itself hint at this complexity. A wide range of neurological phenotypes have been recognized to arise from a primary mitochondrial defect whether it is associated with a mtDNA or nDNA mutation. These include seizures of all types, migraines (and cyclical vomiting syndrome), ataxia, retinal degeneration, deafness, stroke-like episodes (or residual lesions detected by imaging, such as MRI) and the biochemical lesions of lactic acidosis and carnitine deficiency. Therefore to understand defects in oxidative stress in ASD that may have a mitochondrial basis one would need to evaluate the contribution of both the mtDNA and nDNA genes contributing to mitochondrial function. Because of the fact that each tissue is derived from a progenitor cell that inherited a different mix of mitochondria from the fertilized egg there is no ideal single tissue in which to carry out a functional evaluation of mitochondria. Clearly rapidly proliferating cells pose the greatest challenge and while technology may soon make it useful [18], currently it is rarely productive to use routine tests to identify mtDNA mutations in peripheral blood samples. Skin fibroblasts are far better than blood, but skeletal muscle is often selected as the best source, since this post-mitotic tissue hasn't suffered attrition of the most effected cells, those in which the defect will be easiest to observe. It is also important to keep in mind that lactic acidosis and even muscle tissue histology (even at the level of electron microscopy) will not reflect the abnormal genotype in the majority of cases, with observation of the hallmark "ragged red fibers" very rare in young children.

Diseases associated with carnitine deficiency: Carnitine is an essential co-factor in the utilization of fat reserves from body stores during fasting and stress since it plays a key role in the transport of long chain fatty acids into the mitochondria where they undergo beta oxidation in energy production [22, 23, 24]. Carnitine and acyl carnitine derivatives are rapidly lost in the glomerular ultrafiltrate, but free carnitine's ultimate physiological clearance by the kidney is very low because of its active, sodium-dependent resorption in the proximal tubule [25]. The acyl carnitines are poor substrates for transport and hence are rapidly lost in the urine, setting the stage for the "secondary carnitine deficiency syndromes", those that predispose to high concentrations of acyl carnitines and renal wasting [26,27]. Classical examples of inborn errors that produce a secondary carnitine deficiency are the fatty acid oxidation disorders [24, 28], the organic acidemias [29, 30] and defects in the mitochondrial respiratory complexes [31, 32]. No primary defects in carnitine biosynthesis are known to produce disease [33], but primary carnitine deficiency is produced by a rare genetic disease caused by mutations in the *SLC22A5* gene that encodes the sodium-dependent carnitine co-transporter OCTN2 [25, 34], mutations producing a life-threatening carnitine deficiency [34]. In the presence of a block in the mitochondrial respiratory chain, pyruvate is converted to lactate and a lactic acidosis can develop. This is often associated with moderate hyperammonemia since the urea cycle that detoxifies ammonia is found within the mitochondrion. It is also increasingly recognized to be associated with blocks in fatty acid beta-oxidation, also housed in the mitochondrion [35, 36]. Several drugs, but most notably valproate (VPA), also cause a secondary

carnitine deficiency [37, 38]. It is intriguing that this drug that produces this characteristic abnormality in mitochondrial function additionally produces a teratogenic embryopathy and a syndrome in man and rodents with aspects considered to be a pharmacogenetic model of autism (see below).

15q inverted duplication with autism and mitochondrial dysfunction: Chromosomal rearrangements have proven to be critical landmarks in the development of our understanding of genetic disease. Particularly propitious for the discovery of disease mechanism is the paradigm that arises when a characteristic chromosomal rearrangement is recognized in a rare subset of individuals with the disease, as the rearrangement often disrupts the function of a critical gene in the pathway leading to the disease. An important step towards highlighting an energy-deficient endophenotype of autism arose in this context. Our University California, Irvine (UCI) autism center reported that two unrelated children rigorously defined to have autism --- by Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV), Autism Diagnostic Observation Schedule-Generic (ADOS-G) and Autism Diagnostic Interview- Revised (ADI-R)--- in whom we had identified a panel of biochemical signs of mild mitochondrial dysfunction including a secondary carnitine deficiency, also both had an inverted duplication of chromosome 15q11-q13 [39]. Both had uneventful perinatal courses, moderate motor delay, severe hypotonia, periods of moderate to severe lethargy (particularly during an intercurrent illness), elevated urinary lactic acid, elevated serum alanine, lactate and pyruvate and normal electroencephalograms (EEG) and magnetic resonance imaging (MRI) scans. The first child's mitochondrial enzyme assay on muscle showed partial deficiency of respiratory complex III with pronounced mitochondrial hyper-proliferation. The second child's muscle biopsy showed only mitochondrial hyper-proliferation. Assay of the mitochondrial enzymes in skin fibroblasts from both children showed hyper-proliferation and a relative complex III deficiency. Mitochondrial hyper-proliferation reflects a subtle defect in mitochondrial energy production to which the organelles themselves respond by added rounds of division, increasing the number of mitochondria per cell. Karyotype analysis revealed that each patient carried an extra marker chromosome and detailed fluorescent in situ hybridization (FISH) studies revealed that the marker chromosome in both of these patients contains a duplicated segment from the 15q11 – 13 chromosomal region that is at least 22,810Kb in length. Presumably this rare chromosomal anomaly, likely via the tetrasomic dosage of a gene or genes within this interval, produces the disease, including its biochemical and autistic phenotype. This phenotype has now broadened since one of the original subjects, at the onset of puberty, developed intractable seizures and a progressive deterioration of function more characteristic of classical mitochondrial disease. Additionally, since those original cases we have had the opportunity to begin to study several additional cases of this "15q inverted duplication ASD" syndrome, all having the extra chromosomal material derived from the maternal chromosome with the exception of one, quite mildly affected by comparison to the others, that is paternal in origin, suggesting a role of imprinted genes in this classically-imprinted "Angelman /Prader-Willi" region [40].

Rare familial autism with SIDS-like episodes: Within the context of the patients with a weak mitochondrial defect and a recognized 15q inverted duplication, a familial case of autism with sudden infant death syndrome (SIDS) took on new significance. We previously reported [41] three brothers, only children of a healthy non-consanguineous couple, all with the diagnosis of autism by DSM-IIIR criteria and all with an unexplained, long-standing microcytic hypochromic anemia. The oldest brother's Childhood Autism Rating Scale (CARS) classified him as mildly-moderately autistic. He was generally healthy, but from early infancy had poor eye contact and echolalia; his self-stimulatory symptoms began at age three. At age 5, after a 1-day illness, he died suddenly with respiratory arrest and shock, a characteristic lethal presentation of a carnitine-deficient fatty acid oxidation defect. As is generally the case in such disorders, the coroner's autopsy was non-diagnostic [42]. The two younger brothers then aged 2, were monochorionic twins having had normal developmental milestones for the first year. The more severely affected twin had a near-SIDS episode at 4 months, another characteristic presentation of carnitine-deficiency and a fatty acid oxidation defect [42]. Both twins had limited eye contact and delayed speech. They developed perseveration and echolalia at age 2 and were diagnosed within the autism spectrum by age 3. They had mild hyperammonemia and a significantly reduced free carnitine, about 50% normal, with the acyl fraction near normal. Urine organic acids were normal, specifically lacking dicarboxylics. They were started on oral carnitine supplementation and riboflavin and two months later both twins' microcytic anemia had resolved with a reticulocytosis. Evaluation of mitochondrial function from skin fibroblasts showed

mitochondrial hyper-proliferation and an *in vitro* fatty acid oxidation probe indicated an atypical short chain acyl dehydrogenase deficiency similar to that we have seen with a documented lethal mitochondrial respiratory complex II defect [32]. The genetic basis of this syndrome remains to be identified, but the findings suggest that this family may reflect a novel rare cause of familial ASD associated with mild mitochondrial dysfunction and secondary carnitine-deficiency--the deficiency likely via a chronic loss of modestly elevated acyl-carnitine derivatives produced by the primary mitochondrial defect.

Retrospective study of mitochondrial dysfunction in autism: The special cases of autism discussed in the two sections above, both of which had additional features that led to a detailed metabolic evaluation, as well as prior published rare mitochondrial defects found in autism [43, 44] potentially illustrate the paradigm that rare inborn errors may uniquely be able to bring focus to pathways participating in the common, multigenic diseases they mirror. They further are intriguing in view of the epidemiological fact that a significant excess of sudden death, a common feature of the energy deficiency syndromes [45], is observed in autism [46].

The above findings provoked our UCI autism center to carry out a retrospective investigation of cases of more typical autism to see if there were suggestions of a similar biochemical signal of energy deficiency. In this large unselected sample of "typical" patients with autism and no chromosomal abnormality (n=100 from the clinic of Dr P.A. Filipek), the metabolic profile observed for the *group* was a reduced plasma total and free carnitine level ($P<0.001$), moderate hyperammonemia ($P<0.001$) and lactic acidosis, indicated by an elevated alanine ($P<0.001$) and an elevated plasma lactate/pyruvate ratio that was predominated by the pyruvate values since the lactate itself was only modestly abnormal [47]. It is useful to note the large cohort required to provide the analytical power to discern these differences and to recognize that individuals alone are not sufficiently abnormal to be resolved with statistical significance.

Further reinforcing the notion that extreme measures are required to observe the effects of mitochondrial dysfunction in ASD are provided by two subsequent studies. DiMauro and co-investigators [48] explicitly documented mitochondrial DNA mutations in *families* with autism. They showed that the mothers of 4 patients with autism had detectable heteroplasmic levels of the A3243G "MELAS" mutation, but that while they could detect the mutation in two affected brothers with ASD themselves, no mutation could be detected, even using five different tissues in the analysis, in the two remaining affected ASD patients. In this study they also reported an ASD patient with no observable mutation, but with mtDNA depletion, decreased activity in all respiratory complexes and mitochondrial hyper-proliferation. Most recently a large Portuguese population study (n=120) by Oliveira and co-workers [49] revealed that while most patients with autism had an increased lactate / pyruvate ratio, only 20% had a statistically-significant elevated lactate, even fewer (7%) had a functional mitochondrial defect (ascertained in muscle) and none had an identifiable mtDNA mutation.

Endophenotypes of autism, energy deficiency and abnormal calcium signaling: Mitochondria produce a transmembrane proton electrochemical potential ($\Delta\mu_H^+$), via electron transport along the inner membrane's chain of respiratory complexes, which they use to either synthesize ATP or to carry out the coupled transport of other chemical species. The accumulation of calcium is an important mitochondrial function and quantitatively mitochondria represent the major cytosolic calcium reserve. The pharmacogenetic syndrome of Malignant Hyperthermia (MHS) ultimately results from an anesthesia-triggered breakdown in this system via a massive cytosolic calcium release that produces the hallmarks of the syndrome---muscle rigidity directly from calcium-activation of the contractile proteins and then lactic acidosis, hypercapnea, hypoxemia and hyperthermia, as the mitochondria struggle to re-sequester the calcium load. The established proximate causes of the syndrome are a combination of the triggering general anesthetic and inherited dominant susceptibility mutations in one of three known genes: the sarcoplasmic reticulum ryanodine receptor calcium release channel (*RYR1* causing MSH-1 and accounting for the majority of the cases), the sarcolemmal L-type calcium channel (*CACNA1S* causing MSH-5) or the sarcolemmal sodium channel (*SCN4A* apparently causing both MSH-2 and the classical calcium channelopathy phenotype of Hyperkalemic Periodic Paralysis). At the UCI autism center we have observed a family with the endophenotype of ASD, a weak mitochondrial respiratory chain functional deficiency causing mitochondrial hyper-proliferation and MHS, with the later feature thus far, thankfully, only observed in one of the two similarly-affected sibs. While the molecular basis of their syndrome remains to be determined, our hypothesis is that a primary defect lies in mitochondrial function.

Calcium channel mutations in a disorder associated with autism: Recent studies have demonstrated disorders in calcium signaling by two close gene-family relatives of the MSH-5 calcium channel, mentioned above, are found in ASD, with the proof quite strong for *CACNA1C* (the "cardiac-expressed" isoform of the L-type calcium channel) and suggestive for *CACNA1H* (the T-type calcium channel). Keating and coworkers identified a specific missense mutation in *CACNA1C* as the cause of Timothy Syndrome, a multi-system disorder that invariably causes the Long QT cardiac arrhythmia (LQT8), but includes autism as a very common component, along with congenital heart disease, immune deficiency, intermittent hypoglycemia, seizures and an invariant syndactyly [50]. Subsequently this group identified *CACNA1H* mutations in 6 of 461 individuals with autism. While these mutations were located in conserved and functionally relevant protein domains that reduced channel activity *in vitro* and were absent in 480 ethnically matched controls, the mutations did not co-segregate with the ASD phenotype in the pedigrees, showing that the mutations alone were not responsible for the condition [51]. It is also of note that three novel mutant alleles of *SCN1A*, a close gene-family relative of the MSH-2 sodium channel gene mentioned above, have been observed in ASD [52] and that, based upon the role other mutations of this gene play in Familial Hemiplegic Migraine, it too perturbs calcium signaling [53].

New windows into the energy-deficient endophenotype of autism: At this point both the genetic signals and the biochemical signals of this endophenotype are provocative, but far from conclusive. In an attempt to more rigorously test the hypothesis that energy deficiency underlies a component of autism, three new avenues of investigation that will be elaborated upon below seem worthy of pursuit. These include 1) the use of metabolomics---an emerging post-genomics field that relies upon sophisticated measurements of biochemical intermediates to highlight differences that implicate the genetic pathways underlying complex phenotypes----using a measured environmental stressor, aerobic exercise, in an attempt to expand the energy-deficient biochemical endophenotype; 2) the use of physiomics---a technique that allows one to measure differences in abundance (*not* sequence) of hundreds to thousands of gene transcripts using RNA expression microarrays on control and patient samples---- to correlate altered patterns of mitochondrial gene expression with specific energy-deficient endophenotypes; and finally 3) the use of model systems, such as the *in vitro* culture of neuronal stem cells or the model simple neurogenetic organism *C elegans*, to define environmental stressors and candidate disease-causing genes as those perturbing the genetic signaling pathways uniting components of the energy-deficient endophenotype.

Controlled aerobic exercise to expand metabolomic signature of energy-deficiency: Preliminary unpublished studies from the UCI autism center carried out with informed consent in the General Clinical Research Center on controls and subjects with autism aged 12 to 17 proved to be perfectly feasible using an electronically braked cycle ergometer. Each subject was calibrated using a ramp-type progressive exercise test and O_2 uptake, CO_2 production, heart rate and blood pressure were obtained, with the work rate individualized for each participant by finding the work rate corresponding to half of the difference between his anaerobic (or lactate) threshold and his peak rate of oxygen consumption [54]. At a second visit subjects carried out a series of ten two-minute bouts of this constant-work rate cycle ergometry (at their personalized rate, described above) punctuated with 1-min rest intervals and catheter-drawn blood samples and expired breath samples [55] were obtained for analysis. In this small preliminary cohort the C_{16} long chain acyl carnitines were elevated in the ASD cohort at baseline and throughout the protocol, consistent with our retrospective study [47]. Other metabolites were found to differ between the two groups only during the stress of exercise. Additionally distinct outliers among the autistic probands were identified that appeared to represent a unique metabolomic profile, such as one ASD proband with significantly elevated C_8 and C_{10} acyl carnitines throughout the protocol, suggesting a weak defect in the oxidation of the medium-length acyl chains.

Expression microarrays to survey expression pattern of mitochondrial genes: Since the molecular defects that can cause mitochondrial disease can occur not only in the 37 genes of the mtDNA, but also in the estimated thousand-plus mitochondrial genes encoded by the nDNA, a survey of all potential ASD candidate genes producing mitochondrial energy deficiency is very difficult. However gene expression or physiomic defects may potentially be revealed through expression profiles performed on skeletal muscle and/or lymphoblastoid cell line RNA by analyzing the changes in the expression of large numbers of genes by DNA microarray [56]. Simply identifying subsets of gene expression profiles may provide for enhanced molecular endophenotyping in ASD, which should be

particularly valuable in this intrinsically heterogeneous disease.

Valproate-- mitochondrial stressor and serotonergic teratogen in autism: While VPA is perhaps the best recognized cause of drug-induced carnitine deficiency (see above), it is also among the best examples of a teratogen that is able to produce a model of autism, a phenotype produced by only a small number of exogenous compounds. This common seizure medication has been well documented to significantly increase the risk of autism in a large 20-year study of over 600 children exposed to VPA *in utero* in Scotland [57]. Furthermore, embryonic VPA exposure to rodents mimics this effect, causing neuroanatomical, histological and behavioral changes reminiscent of the human disease [58, 59, 60, 61]. Within this model, evidence points to abnormal neuronal stem cell development towards monoaminergic neurons, with defects in serotonin signaling being particularly implicated [58, 62]. The timing of VPA exposure is critical, with a narrow window centered on the stage of early somite development and neural tube closure--- approximately day 12 in gestation of the rat or day 24 in man. The effect is dose dependent and also affected by the polygenic background of the inbred rodent strain, all serving to suggest that this phenomenon models a classic gene-environment interaction in a genetically-susceptible individual—in this case potentially both the teratogen and the mutations targeting mitochondrial function and the critical vulnerable tissue being the serotonergic neuron. This is particularly intriguing in view of the fact that abnormalities in serotonin neurotransmission have been associated with autism, most clearly as hyperserotonemia, one of the best established biochemical markers in autism. This phenotype is now understood to be associated with increased uptake of serotonin into the platelets via the same reuptake transport system responsible for removing this neurotransmitter signal from the synaptic cleft, the selective serotonin reuptake system [4, 63], perhaps explaining the efficacy of drugs that block this system, the selective serotonin reuptake inhibitors (SSRIs) [64], in treating a "hypo-serotonin" behavioral phenotype in ASD.

Serotonin and energy deficiency: In *C elegans* it is known that signaling from the insulin/IGF receptor homolog to the FoxO transcription factor modulates the expression of a battery of genes involved in mitochondrial energetics [65, 66]. Genetic analysis of serotonin-deficient worms implicated serotonin inputs into this signaling pathway [67], a finding we recently were able to explicitly demonstrate [68, 69] by showing that serotonin-deficient mutants, like insulin/IGF receptor mutants, exhibit the FoxO nuclear accumulation characteristic of the altered mitochondrial gene expression and bioenergetics caused by mutants in this pathway. This transcription factor "stress response" arose in both neuronal and non-neuronal cells throughout the body and exogenous serotonin or the SSRI fluoxetine (Prozac ®) attenuated this response. This suggestion of a diffuse change in mitochondrial energetics was reminiscent of the mitochondrial energy deficiency in ASD described above and leads to the hypothesis that one could use this highly-conserved signaling pathway and the elegant genetics of *C elegans* to discover genes that link serotonin signaling and mitochondrial energy deficiency, a class of potentially promising, but otherwise elusive, candidate genes for this endophenotype of ASD. It is also intriguing that this unexpectedly-highlighted pathway in insulin signal transduction now unites together the two major incompletely-explained pediatric epidemics of this generation--- type-two diabetes / metabolic syndrome and ASD--- perhaps hinting at some shared underlying vulnerability.

CONCLUSION

Hints that mitochondrial energy deficiency plays a role in ASD have become more concrete and specific over the past few years and together with the broad approaches to oxidative stress in ASD, it looks like solid leads are emerging that will hopefully soon lead to new approaches to diagnostics and therapeutics that will have impacts for our patients.

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