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DYSREGULATION OF ENDOGENOUS NITRIC OXIDE EXPRESSION AS RELATED TO AIR POLLUTION AS A RISK FACTOR FOR AUTISM

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Abstract- The arginine-nitric oxide pathway is likely to be involved in the neuropathophysiology of autism through dysregulation of nitric oxide (NO) expression during fetal development. This in turn would lead to a cascade of cell signaling effects that impact early brain development and nervous system connectivity, leading to an autism phenotype. Chemical pathways involving nitric oxide expression are influenced by other cell signaling mechanisms, such as the MET tyrosine kinase receptor-ligand system, which is associated with a robust genetic finding of over-transmission of the MET "C" allele in multiplex families having more than one child with autism spectrum disorder (ASD). Dysregulation of nitric oxide expression, it is proposed, will be found in children with autism who have inherited the MET "C" allele based on downstream under-activation of Nitric Oxide Synthase (NOS). This will likely be reflected in significantly different levels of nitric oxide metabolites (NOx or nitrites + nitrates) in the blood and the airways of autistic individuals with the MET "C" allele. Since the Nitric Oxide Synthase (NOS) enzymes that produce nitric oxide are potentially sensitive to allosteric feedback, it is further suggested that nitric oxide air pollution may be a potential environmental risk factor for autism in the genetically susceptible fetus.

Keywords- autism, risk factors, nitric oxide dysregulation, gene-environment interactions, air pollution

Introduction

Autism spectrum disorders (ASDs) are a heterogeneous group of neurologic disorders with complex and varying causes. There is evidence that autism is on the increase [1], raising the question of environmental factors combined with genetic susceptibility to account for the increase. It is challenging to build a theory of autism based on biological abnormalities, given the noted heterogeneity. However, solid genetic findings replicated across cohorts of children with autism show over-transmission of the "C" allele for the MET gene [2-4]. The ASDs involve disruption in connectivity in the nervous system, likely involving the synapse [5,6]. Specific genetic syndromes have been identified in a minority of autism cases, such as Fragile X syndrome or Tuberous Sclerosis, termed "syndromic-autism" [7]. However, for the majority of idiopathic or non-syndromic autism cases, etiology and pathophysiology remain a challenge that is being actively researched and proposed biochemical mechanisms are being sought.

Materials and Methods

A current scientific literature review was conducted on nitric oxide physiology as it relates to published scientific discoveries in the pathophysiology of autism. From this review, scientific argument is made, based on cited research findings, for the necessity of greater investigation into the role nitric oxide metabolism could play in autism spectrum disorders.

Literature Review Results

Nitric oxide (NO) acts as a diffusing messenger important in cell signaling, as a neuroendocrine hormone and as a neurotransmitter [8]. This low molecular weight signaling molecule readily diffuses across membranes down its concentration gradient, resulting in a variety of physiologic and pathologic consequences. NO is important in regulating brain growth and is involved in learning and memory processes and the balance of serotonergic and gabaergic transmitter systems that have been implicated in autism [9,10]. In short, NO is ideally situated to be involved in the neuropathology of autism. NO is formed in cells by a specific set of enzyme isoforms known as the Nitric Oxide Synthases (NOS), which are tightly regulated [11]. Three isoforms have been identified (neuronal or nNOS = NOS I; macrophage-induced or iNOS= NOS II and endothelial or eNOS = NOS III). The endothelial NOS (eNOS) gene is found on chromosome 7, the neuronal NOS (nNOS) gene on chromosome 12 and the inducible NOS (iNOS) gene on chromosome 17. The Nitric Oxide Synthases are cytochrome- P-450-like heme-proteins that depend on molecular oxygen, NADPH, flavins and tetrahydrobiopterin. NOS enzymes catalyze production of NO from the amino acid arginine, forming citrulline and oxidized NADP+ in the process [12]. This reaction can be inhibited by a methyl derivative of arginine and a variety of other molecules. NOS inhibitors can test whether certain physiological phenomena are mediated by nitric oxide, given its many complex biologic roles [13].

In the brain, NO is involved in the formation of cyclic GMP (chemically known as guanosine 3', 5'-cyclic monophosphate or cGMP). This process is enhanced by the presence of glutamate and NMDA receptors [14-16]. The latter are important players in learning and memory based on processes that involve strengthening synaptic connections. NO plays a vital role in such strengthening, related to LTP (long term potentiation) and LTD (long term depression) effects on synaptic strength. Indeed, LTP in the cerebellum and hippocampus results in prolonged change in synaptic strength. This is in part mediated by NO in the cerebellum [17-19]. In the hippocampus, synaptic strengthening is based on pairing of

stimulation to both pre- and post-synaptic neurons and opening of NMDA receptors. The NMDA receptors contain either voltage-sensitive or ligand-activated gates. In order for the NMDA receptor to be fully opened, the post-synaptic neuron must be depolarized by the pre-synaptic neuron in a closely coordinated manner through a feedback molecule. That retrograde feedback molecule is NO [20].

The Met "C" Allele and Autism

In cohorts of non-syndromic autism, a solid genetic finding that has been replicated in at least 6 independent studies is the over-transmission of the "C" allele of the MET gene to children with autism compared to their typically developing siblings, which approximately doubles the risk of autism [2-4]. The MET gene encodes for the MET tyrosine kinase receptor that mediates Hepatocyte Growth Factor (HGF) signaling involved in brain circuit formation, immune function, and gastrointestinal repair. The "C" allele is a functional promoter allele variant that results in reduced gene transcription and thus decreased production of MET tyrosine kinase protein [2]. Kinases are regulator proteins that phosphorylate other proteins. As such, MET tyrosine kinase is embedded in cell membranes, acting as a receptor for its ligand, Hepatocyte Growth Factor (HGF). Activation of the receptor by HGF results in a cascade of diverse intracellular reactions. Such MET receptor signaling is required for sensory nerve development, and HGF promotes axonal growth and survival of both sensory and motor neurons [21]. One chemical pathway that is impacted by the MET tyrosine kinase-HGF receptor-ligand system involves phosphatidylinositol 3-Kinase (PI3K) and Akt (also known as protein kinase B or PKB), which then act on the endothelial form of Nitric Oxide Synthase or eNOS to produce NO. Indeed, it has been shown that Hepatocyte Growth Factor stimulates NO production through eNOS activation by the PI3K/Akt pathway in endothelial cells in the circulatory system [12,22]. An under-expression of the MET tyrosine kinase protein based on inheritance of the MET "C" allele would impact the activity of eNOS and the production of endogenous NO, potentially at critical times and in critical locations in brain development.

Susceptibility risk factors for autism likely reside not just in genetic inheritance, but in gene-environment relationships, with important epigenetic effects. The genetics of the autism spectrum disorders is proving to be very complex, and currently only a handful of candidate genes for autism have been strongly replicated through independent studies. One of the most important, substantiated candidates is the MET "C" allele. A useful model for understanding the interaction of risk factors for autism comes from an integrative threshold concept in which various risk factors have cumulative effects that move the individual toward a threshold beyond which autism results [23]. Since the MET "C" high risk allele is associated with decreased expression of MET tyrosine kinase, it is logical to postulate that other genetic or environmental mechanisms that decrease the downstream effects of MET tyrosine kinase could create additional cumulative risk toward crossing the autism threshold. Hence, genetic or environmental factors that decrease the functioning of eNOS and thereby decrease the expression of NO may also constitute risk factors for autism.

A problem with the theory of inadequate expression of NOS leading to an autism phenotype is that such inadequate expression would not predict an increased risk of seizures, which has been reported in autism [7]. However, given the heterogeneity of the ASDs, it may be that some phenotypes of autism, such as the syndromic forms of autism, are more prone to seizure disorders than other forms of autism which might be relatively spared from seizures. Deficient expression of eNOS, while hard to measure in vivo directly, would likely be reflected in overall abnormal regulation of NO production. Therefore, it would be useful to measure the metabolic products of NO in the blood in the form of nitrites and nitrates in persons with autism. A few studies have reported on abnormal blood nitrites/nitrates in individuals with autism compared to controls. For instance, Sweeten and colleagues [24] studied nitrite plus nitrate metabolites of NO in the plasma along with the cytokines interferon-gamma (IFN-gamma), tumor necrosis factor-alpha and interleukin-1 beta in children with autism compared to gender matched controls. They found that plasma levels of nitrites/nitrates were significantly higher in children with autism, while cytokines levels did not differ between the groups. It is interesting that the nitrite/nitrate levels were abnormally high, not low, in the group with autism. Of note, at least some isoforms of NOS have been shown to be sensitive to allosteric feedback from the enzyme end product, NO [10,25,26]. If blood nitrites/nitrates are typically unusually high in autism, it is possible that this is related to an abnormal sensitivity to NO in the atmospheric environment. NO has the capacity to bind to iron in hemoglobin and is transported in the blood in this fashion [27,28].

Neural Network Theory of Gustafsson

Dr. Lennart Gustafsson, a neural network researcher, is one of the foremost scientists to suggest a role for nitric oxide disturbance in autism [29,30]. His work summarizes studies relevant to neural network and connectionist theories that create computer models of autism based on known science. Indeed, his work is so important to the theme of this review article that it will be summarized in some detail. Individuals with autism do not seem to build adequate, organized cortical feature maps to comprehensively and efficiently extract information from incoming sensory input. Computer simulations have been designed to model self-organization within the nervous system and such studies of self-organization spontaneously generate a columnar organization of neuronal feature maps. This mirrors the actual columnar organization of the cortex, which consists of central processing units called mini-columns. Gustafsson has argued on neural network principles that narrow cortical columns would be inadequate for feature extraction and would therefore be a model of autism [31]. Casanova and colleagues [32] subsequently have shown that at autopsy the neural mini-columns in frontal cortex are indeed narrower in individuals with autism as compared to controls. A related fact is that stable cortical maps require a diffusing messenger like nitric oxide [33-36]. The width of neuronal excitation relates to the width of the neural column and has been found to be dependent on the rate of nitric oxide production, with low production resulting in narrow mini-columns. Thus, Gustafsson hypothesized that inadequate expression of nitric oxide could produce narrow mini-columns and hence an autism phenotype. It is possible that more than one NOS isoform is involved, but this author hypothesizes that eNOS is the most likely isoform to be involved in the pathophysiology of autism. Related to this, high levels of homocysteine inhibit eNOS and genetic homocysteinemia or homocystinuria is associated with mental retardation and speech delay, which are conditions commonly seen in autism [37].

Since inheritance of the MET "C" allele in autism leads to under-expression of the MET tyrosine kinase protein and under-activation of the PI3k/Akt pathway, this would result in less activation of eNOS. In turn, there would be less NO production, consistent with Gustafsson's neural network findings. This would not explain the

high levels of NO metabolites in the blood of some children with autism, though disruption in NO regulation is still implicated by all these findings.

Other research in autism also supports Gustafsson's proposal of deficient NOS expression leading to an autism phenotype. He summarized supporting evidence related to early brain overgrowth, visual sparing and other pertinent characteristics in autism. Several studies have shown early rapid overgrowth of the cerebrum in a significant subset of children with autism compared to controls [38-40]. The overgrowth was typically most pronounced in the frontal lobe but also occurred in temporal and parietal lobes, likely reflecting dysfunction in all these areas. The occipital lobes appeared to be spared. Frontal lobes play a role in social and executive functioning, areas of core impairment in autism. One explanation for early brain overgrowth may be inadequate presence of a growth arresting factor. Several animal studies show that nitric oxide (NO) acts as an arresting factor in fetal brain development for species as diverse as drosophila fly, xenopus frog, and the mouse [41,42]. Peunova and colleagues [43] as well as Enikolopov and colleagues [44] have shown that NO signals cells (i.e. brain cells) in a developing organism when to stop dividing and start differentiating into their final characteristics. Nitric oxide helps mediate this transition from division to differentiation in part because it can suppress DNA synthesis (indirectly) and suppress cell proliferation. Nitric Oxide Synthase inhibitors have been shown to distort brain morphogenesis [43].

Other evidence suggests that NO is important in columnar organization of prefrontal cortex neurons but not in neurons of the occipital lobe, such as in area 17 [45]. This has bearing in autism, where visual functioning tends to remain relatively intact, in contrast to deficits in auditory processing. In fact, many individuals with autism are reported to show strong visual learning and good visual-motor skills [46]. Interestingly, Nitrogen Oxide Synthase (NOS) inhibition does not impair visual or spatial discrimination, but does affect cortical organization and function in the frontal and temporal lobes [47].

With regard to the cerebellum, nitric oxide could be involved in reduced number of Purkinje cells (which act as central processing units), and this is one of the most consistently reported anatomical abnormalities in autism [48]. Reduced Purkinje cell number may be associated with the clinically observed stereotypic motor movements and deficits in motor control that are listed as a diagnostic symptom of autism. NO appears to play a key role in motor learning in cats [49].

Courchesne [40] hypothesized that Purkinje cells are under excitotoxic stress simultaneously with rapid brain overgrowth in autism. Nitric oxide is known to have both a neuro-protective and neurodegenerative role in the brain and to cause excitotoxic stress. Purkinje cells express NO. Logically, then, a decreased level of NO could be related to problematic functioning of Purkinje cells. It has been shown that prolonged inhibition of NOS can aggravate the damage in excitotoxic brain injuries [50].

Another common symptom in autism is clinically significant sleep disturbance [51]. NO plays a role in sleep-wake cycle regulation. The addition of an NOS inhibitor into the pons sleep center of rat brains decreased sleep in the rats [52]. Conversely, the administration of an NO precursor (such as L-arginine) increased sleep. Nitric oxide, through cGMP, also plays a role in melatonin synthesis [53,54]. Melatonin is involved in sleep regulation and low melatonin has been reported in autism [55].

Persons with autism frequently have high pain thresholds or reduced nociception (perception of pain). Nitric oxide facilitates induction of pain perception, so reduced levels of NO could lead to reduced nociception. Both the induction of nociception and its maintenance are facilitated by NO [56,57]. Research has shown that NOS inhibitors exhibit anti-nociceptive properties in rats [58].

Extreme selective eating is a common behavioral disturbance in autism. Disruption of neuronal NOS gene function in snails has been shown to produce reduced feeding behavior, while expression of the neuronal NOS gene appears to be essential for normal snail feeding behavior [59].

DNA Transcription

Although NO alone has no evident effect on transcription, it can act as an amplifier of calcium signals in neuronal cells and thereby indirectly affect transcription. NO and CA 2+ action have to coincide in time for amplification of the calcium signals to occur. Induction of gene activity following NO-amplified calcium action involves protein kinase A-dependent activation of CREB (cAMP Response Element Binding Protein), a transcriptional activating protein [60]. Furthermore, the calcium/calmodulin-dependent kinases (CaMKs) are involved in a large number of cellular responses induced by hormones, neurotransmitters and other signaling. The target proteins for CaMKs bear similarity to each other, and include CREB. It is therefore plausible that the important role that NO plays in amplification of calcium signaling with induction of gene activity in neurons could have significant impact on fetal brain development through the action of CaMKs and CREB.

NO Effects on Dopamine and Glutamine

NOS inhibition (such as by a methyl derivative of arginine) in vivo facilitates dopamine and glutamate transport. Glutamate, through N -methyl D-aspartate (NMDA) receptors, causes an increase in intracellular Ca2+, thereby stimulating NOS and NO production. Reuptake is a major mechanism for inactivation of released glutamate and dopamine. NO inhibits glutamate and H3-dopamine uptake in synaptosomes in rat hippocampus and striatum in vitro [61,62]. If this mechanism also occurs in-vivo, it could operate as a trans-synaptic regulation mechanism, impacting events such as learning and memory. Individuals with autism show a range of impaired learning, from severe mental retardation to problems with abstract reasoning and social referencing despite above-average intelligence.

Infection and Autoimmunity

Prenatal viral infectious disease exposure, such as maternal influenza during gestation, is a risk factor for autism. It is thus particularly pertinent that prenatal viral infection causes alterations in nNOS expression in developing mouse brains [63].

With regard to autoimmunity, a case-control epidemiological study that compared more than 400 children with an autism spectrum disorder (ASD) to a larger control group found only maternal psoriasis had a significant risk association for autism spectrum disorders (ASD) in the offspring [64]. Psoriasis is an inflammatory skin disease with abnormal proliferation of keratinocytes skin cells. Of interest, nitric oxide (NO) is released from keratinocytes at high concentrations and a link has been demonstrated between psoriasis and limited inducible Nitric Oxide Synthase (iNOS) activity [65]. Thus, it is possible that there is a common genetic mechanism for both psoriasis and autism related to dysfunctional NO expression.

Maternal Asthma & Allergy as an Autism Risk Factor

A greater than 2-fold elevated risk of ASD occurs in offspring from mothers suffering 2nd trimester asthma and allergy [64]. NO has been implicated in the pathophysiology of asthma [66,67] and is a known atmospheric pollutant as one of the atmospheric nitrogen oxides (NOx). All three nitric oxide synthase (NOS) isoforms are present and produce NO in the lung. NO metabolites are detectable in sputum, airway aspirates and broncho-alveolar lavage fluid [68]. Intracellularly, NO activates soluble guanylate cyclase to produce cGMP (cyclic GMP or guanosine 3',5'-cyclic monophosphate), which mediates the majority of NO effects. Nitric oxide diffuses into the airway and is measured in the gas phase, with asthmatics showing high levels of NO in their exhaled breath and high levels of NOS II enzyme expression in the epithelial cells of their airways [68,69]. But NO is also a bronchodilator, so increased levels could have beneficial counter-effects to broncho-constrictive stimuli. Likewise, NO has both pro- and anti inflammatory properties and may modulate inflammation in asthma. The increase in NO levels in the exhaled breath of asthmatics seems to be closely related to airway inflammation. Inflammatory cytokines induce and maintain the gene expression of NOS II (iNOS) in airway epithelium. Administration of anti-inflammatory drugs such as corticosteroids result in decreased exhaled NO [70,71]. Why second trimester maternal asthma may be a risk factor for autism in the fetus is unknown. It is not even known if it is the disease itself or potentially the treatment of the disease (for instance, the use of bronchodilator and/or corticosteroid medications) that is the true risk factor. Another possibility is that dysregulation in nitric oxide makes the mother at risk for asthma, and the fetus at risk for autism. Of great pertinence to these issues, it has been found that air pollution (in the form of ozone) alters brain and pituitary endothelin-1 and inducible nitric oxide synthase (iNOS) gene expression in rats and recent work suggests that air pollution is a risk factor for cerebrovascular and neurodegenerative disease in humans [72]. More will be said on air pollution and autism risk at the end of this article.

Synaptogenesis and Cell Adhesion

Mutations have been found in two X-linked genes encoding neuroligins that affect cell-adhesion molecules localized at the synapse in autistic subjects, suggesting a possible defect in synaptogenesis [73]. More will be said later on variations related to genes that code for cell-adhesion molecules, which were recently found to be over-transmitted to children with autism.

Redox Imbalance

Redox or reduction-oxidation state affects synaptogenesis and apoptosis processes, raising the issue of whether redox imbalance in autism (potentially related to dysregulation of NO) may contribute to defective synaptogenesis. For good health, reactive oxygen species (ROS), other free radicals and anti-oxidants should remain in balance, with overabundance of ROS and free radicals causing oxidative stress. Oxidation is the loss of electrons or hydrogen and can damage cellular lipids, proteins and DNA. One study showed that oxidative stress with resultant poor cellular energy balance and excitotoxicity occurs in children with autism verses controls [74]. This study measured plasma concentrations of metabolites in the methionine trans-methylation and trans-sulfuration pathways. Subjects with autism had comparatively significantly lower baseline concentrations of methionine, SAM (S-Adenosyl Methionine), homocysteine, total glutathione and related molecules. There were significantly higher concentrations of SAH (S-Adenosyl Homocysteine), adenosine and oxidized glutathionine. This metabolic profile is consistent with impaired capacity for methylation, due to the significantly lower ratio of SAM to SAH. Increased oxidative stress was shown by the significantly lower redox ratio of reduced glutathione to oxidized glutathione in the children with autism. A targeted nutritional intervention trial with folinic acid, betaine and methylcobalamin (Vitamin B 12) was effective in normalizing the metabolic imbalance in children with autism on brief follow-up. The authors concluded that an increased vulnerability to oxidative stress and a decreased capacity for methylation may contribute to the manifestations of autism.

Peptides, Oxidative Stress and Autism

Adrenomedullin (AM), a small amino acid peptide hormone, induces vaso-relaxation by activating adenylate cyclase and also by stimulating NO release. AM immune reactivity is present in the brain, consistent with an additional role as a neurotransmitter. NO and AM appear to function in the regulation of many neuro-developmental processes. Zoroglu and colleagues [75] found that mean values of plasma total nitrite and AM levels in a group of children with autism were significantly higher than control values. This was similar to the finding of higher plasma total nitrite in autism noted by Sweeten and colleagues [24]. There was no correlation between total nitrite and AM levels. A later study by Zoroglu et al. [76] assessed the changes in superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities and thiobarbituric acid-reactive substances (TBARS) levels in plasma as well as NO levels in red blood cells (RBC) in subjects with autism compared to matched controls. The autistic group showed increased RBC NO levels ($p<0.0001$) and plasma GSH-Px activity ($p<0.0001$), with unchanged plasma TBARS levels and SOD activity. These findings further support increased oxidative stress, in part related to NO levels, and altered enzymatic anti-oxidants as relevant to the pathophysiology of autism.

Immunologic and Neuro-Immunological considerations

There is growing consensus in the scientific community that the disorder of autism involves interaction of genetic, environmental and neuro-immunological mechanisms. Supporting this consensus are studies that have shown alteration in the balance of peripheral blood T-helper (Th1 and Th2) cells and increased frequency of auto-antibody products in persons with autism [77,78]. Another study showed that controls have greater plasma Immunoglobulin E (IgE) than subjects with autism [79]. Nitric oxide expression is correlated with higher IgE levels [80], thus the lower levels of IgE in the group with autism may suggest dysregulation of NO production. Additional data suggests that, under the age of 5, the immune system in children with autism does not efficiently process and present antigen as compared to normal controls. Here again, nitric oxide plays a role, as it is involved in antigen processing [81].

Pardo and colleagues [82] documented the presence of neuroglial and innate neuroimmune system activation in postmortem brain tissues of eleven persons with autism compared to controls. These researchers found on-going neuro-inflammatory processes in the cerebral cortex, white matter and especially in the cerebellum within the Purkinje cell layer in the brains of persons with autism. There was marked activation of microglia and astroglia. Immunocytochemical staining for Major Histocompatibility Complex (MHC) markers also showed marked microglial activation, again prominent in the cerebellum but also in cortical regions and white matter (this was

highly significant at p<0.0001). They noted that a non-genetic, neurotoxic or environmental process could produce the observed neuronal and cortical abnormalities, to which the marked neuroglial reactions may only have been secondary responses. Vargas and colleagues [83] characterized the cytokine profile of the cerebrospinal fluid (CSF) in six patients with autism as compared to CSF from a pool of donors without central nervous system pathology or inflammatory disorders. A marked increase in subsets of cytokines and chemokines involved in innate immune responses was found. These cytokines play important roles in immune-mediated processes and their presence in the CSF of persons with autism may reflect ongoing inflammatory reactions associated with neuroglial activation and/or neuronal injury. The cause of the increased inflammatory molecules remains unknown, but it is known that nitric oxide modulates microglial activation [84,85]. In the future, CSF cytokine profiling may assist in the diagnosis and in following the clinical course of autistic disorders.

Neurogenesis, Synaptic Plasticity, Cell Adhesion and Inflammation

It is intriguing that NO plays a role not only in embryonic neurogenesis but also potentially in adult neurogenesis in rodents [86,87]. It is therefore possible that NO dysregulation can not only affect the brain development of a fetus, but can continue to affect brain functioning from childhood through adulthood.

Synaptic plasticity is an important characteristic of brain functioning. The family of MAGUK proteins (membrane-associated guanylate kinases) affects the clustering of different NMDA receptor subunits and thus may be involved in synaptic plasticity, possibly mediated in key ways by nitric oxide [88]. Additionally, neural cell I adhesion molecules (NCAMs) modulate lipopolysaccaride-stimulated production of NO in cultured glial cells, and thus neuron-glia interactions via NCAMs play an important role in regulating the activities of glial cells via nitric oxide [89]. This is important in light of findings of glial inflammation in certain brain areas in autism [82,90].

Cell adhesion molecules have important roles in brain development and function. Genetic variations between the cadherin 9 and cadherin 10 genes that produce certain NCAM proteins were found to be significantly associated with autism [91]. While the protein products of these particular cadherin genes have not yet been shown to interact with nitric oxide metabolism, other cadherin proteins, such as vascular endothelial cadherin, do impact nitric oxide production [92,93]. Endothelial nitric oxide synthase (eNOS) activity is affected by cell adhesion, as activation requires localization of eNOS at intercellular junctions with cell to cell contact [94]. On the flip side, eNOS is also a determinant of endothelial junctional integrity [95].

Mitochondrial Disease in the Autism Spectrum Disorders

A number of reports have indicated an association between autism spectrum disorders and mitochondrial dysfunction [96-101]. These studies cite disorders of mitochondrial oxidative phosphorylation related to enzyme- or mutation-defined electron transport chain dysfunction as important in certain uncommon autism phenotypes. A review of children with autism spectrum disorders later shown to have mitochondrial dysfunction noted that 24 of 25 patients had one or more major, uncommon clinical abnormalities in autism, including easy fatigability, marked delay in early gross motor milestones, unusual patterns of regression and some red-flag abnormalities for certain common blood tests [96].

Mitochondria may be one of the major targets for NO, since they are rich in iron-sulfur chemicals and iron-heme proteins. Nitric oxide (NO) and its derivative peroxynitrite (ONOO-) inhibit mitochondrial respiration in brain nerve terminals [102]. This inhibition appears to contribute to both the physiological and cytotoxic actions of NO. In competition with oxygen, nanomolar concentrations of NO rapidly and reversibly inhibit cytochrome oxidase in the mitochondrial electron transport chain.

Reactions have been demonstrated in yeast cell studies of NO with cytochrome c [103]. It is likely that such reactions are relevant to mitochondrial metabolism of NO. Ferricytochrome c in the mitochondria can act as a reversible sink for excess NO. Additionally, reduction of NO to NO- by ferrocytochrome c may play a role in the irreversible inhibition of mitochondrial oxygen consumption by peroxynitrite.

A mitochondrial Nitric Oxide Synthase (mtNOS) exists that is dependent on calcium (Ca2+) and is related to mitochondrial bioenergetics [104]. This mitochondrial NOS has possible involvement in apoptosis related to elevated Ca2 + levels, and it is known that certain areas of the brain show apoptosis in autism, particularly in the cerebellum. Uptake of Ca2+ by mitochondria triggers mtNOS activity and causes the release of cytochrome c from isolated mitochondria. This release of cytochrome c was prevented by NOS inhibitors, a superoxide dismutase mimic and a peroxynitrite scavenger.

Reelin and Nitric Oxide

Genetic linkage studies have yielded inconsistent results regarding Reelin gene alleles and susceptibility to autism spectrum disorders [105,106]. Nonetheless, findings of reduced Reelin protein and mRNA give supportive evidence for impairments in the Reelin signaling system in frontal lobes and cerebellum on post mortem exams of brains of persons with autism compared to controls [107]. Reelin is an extracellular matrix protein that has Apolipoprotein E2 (ApoEr2) as its receptor and is responsible for correct lamination of the brain during the embryonic period. Furthermore, both nitric oxide (NO) and Reelin modulate neuronal plasticity in developing and mature synaptic networks. Reciprocal signaling has been found between Reelin, the NO-sensitive guanylyl cyclase (NOsGC) and ApoEr2/nNOS expressing neurons in the olfactory bulb [108].

Discussion

To briefly summarize above pertinent points**,** nitric oxide (NO) and the Nitric Oxide Synthase (NOS) isoforms that catalyze production of NO are likely suspects for key involvement in the pathophysiology of autism. This is based on diverse impacts of NO on so many aspects of nervous system biology, ranging from influence on the width of cortical mini-columns, to redox balance, contribution to oxidative stress, involvement in neuron cell injury or death and neuro-inflammatory processes, actions as a neurotransmitter and cell signaling molecule, impact on other neurotransmitters, involvement in neuronal differentiation, synaptogenesis, mitochondrial functioning and brain cell plasticity and finally potential downstream effects on eNOS related to reduced transcription of the MET gene when the promoter “C” allele, a factor that doubles the risk of autism, is inherited.

Toward Testable Hypotheses

Converging streams of evidence call for experimentation related to

the regulation of the arginine-nitric oxide pathway that produces NO in the pathophysiology of autism. Because over-transmission of the MET "C" allele to children with autism has been such a robustly replicated genetic finding, and a cell signaling pathway activated by MET tyrosine kinase activates the epithelial Nitric Oxide Synthase (eNOS), it is natural to hypothesize the co-occurrence of inheritance of the MET "C" allele with other abnormalities in nitric oxide regulation as being important in autism. This could occur directly from downstream effects of the MET "C" allele decreasing expression of MET tyrosine kinase, but potentially other mechanisms disrupting nitric oxide expression could co-occur. Empirical questions are raised regarding a possible correlation between MET "C" allele inheritance, inheritance of genes that regulate the expression of NOS (especially eNOS) and a number of physiological processes or products related to nitric oxide that vary in ease of measurement. These include but are not limited to mitochondrial respiration (difficult to measure in vivo but could be studied in post-mortem brain tissue), measures of total body NO production and measures of nitric oxide in exhaled breath and levels of plasma and RBC NOx (relatively easy to measure). While there are perhaps close to 200 genes likely to be involved in NOS regulation [109] which makes testing of such genetic associations with MET "C" allele inheritance challenging (though still worthwhile), it is readily feasible to test the correlation of MET "C" allele inheritance in autism with plasma and RBC NOx measures. However, it is important to control for diet as a confounding factor, as a growing body of research shows that dietary intake does help regulate nitric oxide synthesis, especially the level of arginine intake in protein in the diet [110]. Such dietary factors can be controlled through detailed food logs on subjects correlated with plasma arginine levels in order to reveal the inherent interactions of the genetic metabolic pathways of interest. Nitric oxide metabolism could be more directly tested in newborn biological samples of infants at high risk for autism (such as infant siblings of a child with autism), bypassing the need to account for the influence of dietary factors.

Many other hypotheses related to regulation of NO metabolism emerge for further testing. As an example, if high blood NO metabolite levels (nitrites/nitrates) were found to correlate with children with autism and the MET "C" allele, this would be difficult to account for based on the likely impact of decreased MET tyrosine kinase expression leading to decreased activity of downstream eNOS. However, one explanation of high blood NO metabolite levels in autism could be that children with autism are more sensitive to nitrogen oxides in air pollution as the source of the high blood levels. It is possible that increased absorption of environmental NO could allosterically feedback and further down-regulate the Nitric Oxide Synthase (NOS) isoforms (including eNOS), which together with the MET "C" allele effects cumulatively could lead to inadequate NOS activity to such a degree at critical times and locations in brain development that the autism threshold is crossed (supporting Gustafsson's proposal).

If further research substantiates abnormalities of NOS regulation and NO expression in the autism spectrum disorders, a potential likely environmental toxin is excessive NOx in the air, and especially nitric oxide itself. NOx is a form of greenhouse gas pollution, resulting from industrialization such as coal-burning plants, automobile exhaust, other industrial sources and cigarette smoking [68, 111,112]. Related to the latter fact, maternal cigarette smoking during pregnancy could be a risk factor for autism. Mouse and monkey studies on the fetal effects of maternal chronic exposure to high-NO content in the air during pregnancy would also be important. More detailed epidemiological studies of the relative risk of autism in urban verses rural areas, ideally accompanied by specific measures of air pollution that include NOx air pollution in the geographic areas where the dyad of the mother and her child with autism lived during the pregnancy and during the first few years after birth of the child would need to be conducted.

Nitric Oxide, Toxicity and Brain Cell Death

Nitric oxide plays an important role in neuronal injury and cell death and hence could be related to the loss of Purkinje cells in the cerebellum of autistic individuals. One mechanism of nitric oxide-mediated neuronal cell death is related to thiol dependency [113]. Most neuronal cells, especially those lacking the ability to generate NO (which constitutes the bulk, some 98% of neurons), are vulnerable to damage by reactive nitrogen species and/or reactive oxygen species. Inhibition of NO synthesis has been shown to reduce neuronal injury mediated by glutamate, NMDA receptor activation, down-regulation of Superoxide Dismutase (SOD) and other triggers. Further support for the role of NO in neuronal injury comes from animal modes utilizing (NOS1) knockout mice that were found to be resistant to stroke, NMDA activation and various mitochondrial neurotoxins. On the other hand, expression of NO can also be neuroprotective at times, and the dual role of NO in injury verses protection is not well understood but is actively being researched.

There are many potential target biochemicals that NO may react directly with in the cell, including heme-containing proteins, iron- and sulfur-containing proteins, and reduced thiols, such as glutathione [113,114]. These reactions are utilized primarily in regulation of proteins and in signal transduction and are reversible. As previously noted, mitochondria are one of the major targets for NO, since they are rich in iron-sulfur chemicals and iron-heme proteins. The interaction of nitric oxide (NO) with mitochondria has pathological significance but also presents as a potential mechanism for the regulation of mitochondrial function. In fact, NO may affect mitochondria by reacting with low-molecular weight thiols like glutathione and with larger protein thiols, having a range of implications for cell function based on redox signaling. Because the mitochondrial respiratory chain is a major source of reactive oxygen species (ROS), mitochondria have a range of antioxidant defenses, with the thiols being especially important [114]. The redox state of mitochondrial thiol proteins potentially impacts health. Nitric oxide may be important in affecting mitochondrial redox state as it can modulate respiration at Complex IV. Furthermore, a mitochondrial Nitric Oxide Synthase (mtNOS) exists. Nitric oxide can lead to modification of protein thiols by several mechanisms that have been linked to consequent alterations in protein function. Such changes in redox state and thiol proteins may affect mitochondrial function and conceivably communicate redox status to the rest of the cell [115].

With regard to cell toxicity, nitric oxide reacts with superoxide to form peroxynitrite (ONOO-), a higher redox potential oxidant and nitrating species. In contrast to NO, the reactivity of peroxynitrite with proteins and mitochondria may be detrimentally irreversible. Reduced thiols may be critical reactants for NO and peroxynitrite. Because the reactions of NO are generally reversible, while the reactions of peroxynitrite are generally not, these two free radicals tend to produce different types of cell toxicity. In nitrosative verses oxidative stress, largely necrotic injury to neurons occurred after peroxynitrite (ONOO-) exposure in contrast to apoptotic-like neu-

ronal morphology after excess NO exposure [113]. Post-mortem studies of the brains of persons with autism have shown apoptotic cell loss of Purkinje cells in the cerebellum, not necrotic loss. Thus, it is possible that excessive formation of reactive oxygen and especially nitrogen species may be involved in Purkinje cell damage in autism. Brain tissue studies from autism tissue banks could address this question.

Conclusions

There are some 80,000 commercially important chemicals available in the world and any of a number of them might constitute a significant environmental risk exposure for a fetus genetically susceptible to autism. Yet most of these commercial chemicals are untested for toxicological effects [116]. Deciding which chemicals and environmental pollutants to research for possible autism risk factors should be guided by scientific principles, based on likely neurological and developmental effects of the chemicals, evidence of genetic risk factors being related to a chemical exposure in some manner and/or epidemiological studies that demonstrate increased relative risk of autism in exposed groups verses non-exposed controls. For instance, organophosphate and neo-nicotinoid insecticides affect the balance of excitatory and inhibitory neurons in the brain, so they are worthwhile to investigate in studies of exposure risk related to autism outcome. Chemicals that impact important brain transmitters implicated in autism, such as gamma aminobutyric acid (GABA), are also likely candidates, especially since certain GABA receptor genes have been implicated in some families with autism [117]. Additionally, as this article has summarized, chemicals such as nitric oxide that affect metabolic pathways involved in basic cellular processes like calcium signaling are also likely suspects. Given that the autism spectrum disorders are reported world-wide and are on the increase, chemicals that are increasing in our environment and are widely distributed are particularly attractive targets for study. In this regard, it is important to recognize the increase in NOx air pollution associated with the general overall increase in green-house gases from our industrial age. NOx air pollution gets readily and widely distributed with atmospheric currents. Combining this knowledge with biologic facts cited in this paper about the role of nitric oxide as a neurotransmitter and cell signaling molecule makes nitric oxide and NOx air pollutants important targets to investigate with respect to autism risk.

Consequences on Models of Autism Risk

Intensive research is gradually revealing solid candidate genes for autism, such as the over-transmission of the MET “C” allele and common genetic variants related to cell adhesion molecules. However, the autism spectrum disorders are complex multi-genetic disorders and the search for other candidate genes continues. Since up to one half of the human genome of about 100,000 genes may be dedicated to brain development, there are myriad possibilities for “neuro-gene” variants in autism. However, recognizing the fundamental role that nitric oxide plays in brain development, including in the development of the cortical mini-columns, in synaptogenesis and in mitochondrial bioenergetics, may help to narrow the search. Thus, the genes that code for Nitric Oxide Synthase (NOS) and/or genes and their protein products that are involved in NOS regulation are potentially implicated in autism-risk. It has been estimated that there are approximately 200 genes involved in NOS regulation [109]. A body of research is evolving on regulation of these genes [118].

Significant progress is unravelling the complex genetics of the autism spectrum disorders, particularly as related to interactions with environmental exposures. There is a growing body of evidence that air pollution exposure (such as via living close to a freeway) is a significant risk factor for autism [119,120]. Indeed, nitrogen dioxide is one important constituent of air pollution that together with other air pollutants have shown to be risk factors for autism [119]. Thus, improving air quality potentially could decrease the incidence of autism. Understanding what if any role nitric oxide metabolism plays in the neuro-physiology of the autism spectrum disorders can lead to better testing of the role NOx air pollutants play as environmental risk factors. It is mind-boggling to contemplate that a major air pollutant includes nitric oxide, which is also a cell signaling molecule that rapidly crosses cell membranes, is involved in fetal brain development and is a neurotransmitter. Furthermore, human beings are exposed to this pollutant and other nitrogen oxides with every breath they take, and such pollutants are easily distributed world-wide, just as autism is a world-wide problem.

If inadequate production of endogenous NO during critical periods of brain development is definitively shown to play a role in the pathophysiology of autism, then it is possible that medications that either up-regulate NOS or act as nitric oxide donors would be worthwhile to experimentally test in the treatment of autism. This should be done carefully, with testing in animal models of autism first, unless there is already extensive pediatric pharmacologic knowledge about a particular medication. The statins used to treat high cholesterol up-regulate eNOS, but their safety during pregnancy and in very young children has not been established [121]. A number of novel NO donor drugs have been developed as potential alternatives to conventional nitrates first used a century ago to relieve angina [11]. Experimental medications, such as HCT 1026 (a nitric-oxide donating derivative of flurbiprofen) would definitely require animal testing first, followed by establishment of safety profile before being considered for use in very young children.

Finally, high levels of atmospheric nitric oxide and NOx, as risk factors for autism, constitute a major world-wide public health issue. This highlights the necessity for tighter controls on air pollution and the need for environmental clean-up to better support the health and development of our children. Such an effort would hopefully lead to the prevention of at least some types of non-syndromic autism and is one more powerful reason to combat global warming related to human activity and our industrialized age.

Abbreviations

ASD(s) : Autism Spectrum Disorder(s).

NO : Nitric Oxide.

NOS : Nitric Oxide Synthase

eNOS : endothelial NOS.

iNOS : inducible NOS.

nNOS : neuronal NOS

mtNOS : mitochondrial NOS.

NOx : Nitric Oxides and also used for nitric oxide metabolites.

cGMP : cyclic GMP or guanosine 3’,5’-cyclic monophosphate.

NMDA : N-methyl D-aspartate.

LTP : Long Term Potentiation.

LTD : Long Term Depression.

HGF : Hepatocyte Growth Factor.

PI3K : Phosphatidylinositol 3 Kinase.

Akt : also known as protein kinase B or PKB.

NADPH & NADP+ : Nicotinamide Adeninine Dinucleotide Phosphate.

IFN gamma : Interferon gamma.

CREB : cAMP Response Element Binding Protein.

CaMK : Calcium/calmodulin-dependent protein Kinase.

SAM : S-Adenosyl Methionine.

SAH : S-Adenosyl Homocysteine.

redox : reduction/oxidation.

ApoEr2 : Apolipoprotein E2.

AM : Adrenomedullin.

SOD : Superoxide Dismutase.

GSH : Px-glutathione peroxidase.

TBARS : Thiobarbituric Acid-reactive Substances.

RBC : Red Blood Cell.

NCAM : Neuronal Cell Adhesion Molecule.

Th 1 & 2 : T-cell helper 1 & 2.

IgE : Immunoglobulin E.

MHC : Major Histocompatibility Complex.

CSF : Cerebral Spinal Fluid.

MAGUK : membrane-associated guanylate kinases.

NOsGC : NO-sensitive guanylyl cyclase.

ROS : Reactive Oxygen Species.

GABA : Gamma Amino Butyric Acid.

Conflict of Interest: None Declared.

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