A Possible Central Mechanism in Autism Spectrum Disorders: Interaction of Activated Microglia, Excitotoxicity, Reactive Oxygen and Nitrogen Species, Lipid Peroxidation Products and the Role of Elevated Androgen Levels in Autism Spectrum Disorders

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Abstract

The autism spectrum disorders are a group of related neurodevelopmental disorders, which have been increasing in incidence since the 1980s. While a large mass of information and data has been forthcoming, still a central unifying mechanism has not been offered. A review of the studies on autism spectrum disorders disclose a number of findings that are interconnected.

Most important appears to be disruption of brain cell calcium homeostasis by a number of events, such as excitotoxicity, androgen excess and elevations in inflammatory cytokines. Free radical generation and lipid peroxidation is a common event in examined cases of autism spectrum disorder.

Recently, researchers have discovered high levels of androgens in a group of autistic children and that pharmaceutical lowering of androgen levels can produce rapid and dramatic improvement in a number of cases. Several studies have shown worsening of neurological injuries under conditions of high androgen levels. In addition, high androgen levels have been shown to trigger calcium accumulation in neurons through a special membrane receptor.

Likewise, a number of researchers have found rapid improvement in symptoms following anti-inflammatory treatments or dietary changes. What all these events have in common is that they magnify the excitotoxic process, a major mechanism for CNS injury due to a number of insults, including hypoxia/ischemia, heavy metal poisoning, trauma, infections, seizures and hypoglycemia.

Many have noted an association between an increase in the number of vaccines added to the immunization schedule coincident with the rise in autism cases. An abundance of research has shown that systemic immune activation can trigger brain microglial activation, the resident immune cell type for the central nervous system (CNS). It has also been shown, that when these microglia are primed by prior stimulation, the intensity of the immune reaction is greatly magnified. Subsequent immune stimulation turns this into a chronic state of activation.

Others have noted the connection to high mercury burdens in the autistic child secondary to mercury containing vaccines (thimerosal) and from other sources. Mercury is a powerful activator of microglia, even in very minute doses. In addition, mercury inhibits a number of energy-generating enzymes systems, triggers free radical generation and lipid peroxidation, inhibits a number of antioxidant systems and inhibits the glutamate transport proteins, also at very low concentrations.

Because the majority of the vaccinations are given during the period of most rapid brain growth, such hyperimmune brain activation risk disrupting neurodevelopment, especially for higher cortical functions. This has been repeatedly shown in experimental animals. Intimately connected with CNS microglial activation is the release of powerful excitotoxins, glutamate and quniolinic acid. This is especially so since glutamate plays a critical role in brain cell migration, differentiation, synaptic stabilization and pruning. The level of glutamate, which fluctuate throughout neurodevelopment, is critical. Here I show that altering these levels by way of immune alterations and other mechanisms, plays havoc on the developing nervous system.

Introduction

Autism spectrum disorders are an increasingly common group of neurodevelopmental disorders without an as yet clearly defined cause. This spectrum of disorders is characterized by a collection of neurobehavioral and neurological dysfunctions often occurring before age 36 months, which include a loss of eye contact, deficiencies in socialization, abnormal theory of mind function, language dysfunction, repetitive behaviors and some difficulties with executive prefrontal lobe functions.[1,2]

The disorder has a prevalence of males to females of 4:1. A regressive loss of developmental skills occurs in 30%, most often between the ages of 18 to 24 months. It has also been noted that autistic boys are more likely to experience an early onset of puberty.[3-5] Recent epidemiological evidence indicates a rapid rise in the prevalence of autism, with a 1 in 150 to a 1 in 160 incidence.

Neuropathological studies have shown abnormalities in the architecture of the autistic brain affecting cortical, subcortical, limbic and cerebellar structures.[6,7,8] One of the most consistent findings has been hypoplasia of the inferior vermis of the cerebellum with variable, but substantial loss of Purkinje cells in the cerebellar cortex.

The bulk of the evidence indicates that immune factors play a major role in these disorders.[9-11] Likewise, abundant evidence implicates mercury neurotoxicity from previously high levels of ethylmercury used as a preservative (thimerosal) in a number of childhood vaccines, as well as other sources of mercury.[12,13]

A host of other observations related to autism spectrum disorders have been aired, including abnormalities in organic acids, opoid-like substances from gliadin and gluten metabolism, intestinal dysbiosis and trace element imbalances. A strong genetic influence is also known to exist.[14]

Neuroscience has discovered one mechanism that explains most of the finding in autism spectrum disorders, and that is the excitotoxic cascade. New studies have linked a number of seemingly unrelated events to this cascade, such as immune activity, neurohormone abnormalities and a host of biochemical events.[15,16] Examination of the pieces to this puzzle, demonstrate that most fit well into this mechanism.

The Excitotoxic Cascade

In 1957, Lucus and Newhouse discovered that monosodium glutamate (MSG) exposed rats developed degeneration of the inner ganglion layers of the retina.[17] John Olney in 1969, discovered that the food additive monosodium glutamate (MSG) could produce delayed neuron death when animals were fed the substance in higher concentrations.[18] He not only observed destruction of the animals' retinal neurons, but also destruction of selected nuclei in the hypothalamus and other brain structures. He coined the name excitotoxin, based on the early observation that the neurons seemed to excite themselves to death in a delayed manner.

The glutamate receptor system consists of three ionotropic receptors (NMDA, AMPA and kianate) and three metabotropic receptors types, with a number of cloned subtypes for each receptor.[19] Arrangement and expression of the various subtypes determines function. It is also known that glutamate receptors are not static, but rather can be induced.[20]

The various glutamate receptors differ in structure and physiology, with the NMDA receptor operating via a voltage gated calcium channel and non-NMDA receptors (AMPA/kainate) operating via intracellular calcium signaling. It is the patterns of subunit assembly within the various glutamate receptors that produce the regional differences in the brain's response to glutamate stimulation.[21] Metabotropic receptors act upon G-protein membrane signaling systems. AMPA type glutamate receptors alter their membrane expression by endocytosis, thus increased glutamate stimulation increases their synaptic expression.[22]

The NMDA receptor, which plays a major role in brain development, when activated opens a calcium channel and the rise in intracellular calcium activates a number of enzymes and cell signaling molecules, including iNOS, protein kinase C, phospholipases A2 and the eicosanoid cascade. Excessive stimulation can trigger two reactions that can lead to neuron death. One is the classic excitotoxic cascade and the other is glutamate-induced oxidation via glutamate inhibition of the X_C^- antiporter.[23] The latter mechanism involves suppression of cystine uptake with a resulting reduction in glutathione generation.

Protection against excitotoxicity is highly energy dependent. Norvelli et al. demonstrated that inhibitors of oxidative phosphorylation allowed glutamate to become excitotoxic at concentration that normally produced no toxicity.[24] This explains the triggering of excitotoxic neuron destruction during hypoglycemia without glutamate elevation.[25]

Since the original observation by Olney, neuroscientists have discovered that the brain contains abundant glutamate receptors and that excessive stimulation of these receptors can initiate widespread destruction of a number of brain structures.[26-28] It has been shown that glutamate plays a critical role in the development of the central nervous system.[29-34] Marret and co-workers demonstrated, using glutamate receptor agonist ibotenate, that NMDA receptor excitotoxicity could arrest neural migration in a developing hamster brain.[35] They found intracortical and molecular layer heretopias, subcortical and intracortical arrest of migration and ectopias in the molecular layer of the neocortex. At higher doses they found periventricular and band hererotopias. The effects on brain architecture and migration patterns were dose-dependent. The NMDA agonist produced arrest at all levels of the radial migratory corridors (germinative zone, white matter, cortical plate and molecular layer).

Others have implicated NMDA receptors in migration of granule cells in rat cerebellar slices.[36] This is of particular interest because a number of studies have shown the cerebellum to be one of the most involved areas in autism spectrum disorders.[37,38] Komuro and Rakic have demonstrated that fluctuations in intracellular calcium secondary to voltage-gated NMDA receptor channel activation controls the speed of granule cells migration, with peaks speeding up migration and troughs slowing down migration.[39] It has also been shown that termination of granule cell migration is triggered by a fall in Ca^{2+} levels and that patterns of calcium fluctuation determine migrations to different cortical layers.[40] Because timed peaks and troughs of glutamate brain levels are critical to CNS development, factors altering these levels can have devastating effects on brain development and maturation.[41]

Prolonged behavioral abnormalities have also been demonstrated with glutamate exposure during development of the brain.[42-44] Of special interest is the finding by

Dubovicky and co-workers who found that raising glutamate brain levels in the early postnatal period in rats produced defects in adjusting to a new environment 21 and 60 days later, something also found in autistic children.[45] In addition to this study, others have found that the toxicity and behavioral effects of neonatal and early postnatal glutamate exposure was significantly more evident in males, with little effect found in the females.[46] Also of interest, the affected males showed little social interest in littermates, had defects in novelty and perceptual mechanisms and an inability to focus attention, again, things common to autistic children. These behavioral changes were prolonged, lasting well into adulthood.

Besides the effect on neural migration and maturation, excess glutamate can trigger destructive reactions that can result in a loss of dendrites, synaptic connection and even neurons. Vargas and co-workers described degeneration in cortical, subcortical and cerebella in a series of 11 autopsied autistic patients from ages 4 yrs to 45 yrs.[48] Most consistent in reported neuropathological studies of autism has been the finding of extensive loss of Purkinje cells in the cerebella of autistic patients.[49,50] Several studies have shown that cerebellar deficits, in conjunction with its fronto-limbic connections, can explain a number of the behavioral and learning problems seen in autism.[51-53]

With glutamate being the most abundant neurotransmitter in the brain, a complex system exists to protect neurons from excitotoxic destruction. This consist of the glutamate transport proteins (EAAT1-5), glutamine dehydrogenase and glutamate decarboxylase. Also involved is the glutamate/cystine X_c^- antiporter.[54] The latter being involved in glutamate exchange, with the intracellular movement of cystine used in glutathione synthesis. Excess extracellular glutamate inhibits cystine transfer, resulting in a fall in intraneuronal glutathione levels.

A loss of glutathione is of special importance because oxidative stress plays a major role in the neurotoxic effect of excitotoxicity. Excessive activation of glutamate receptors can initiate the generation of a number of free radicals including hydroxyl, peroxyl and peroxynitrite.[55] Peroxynitrite is especially damaging to the mitochondria, resulting in enhanced ROS formation.[56,57] In addition, a number of lipid peroxidation products are produced during excitotoxicity, the most harmful being 4-hydroxynonenal.[58,59] Oxidative damage has been shown to occur in the developing brain and is exacerbated by glutamate excitotoxicity.[60]

Microglia, Neurogenesis and Neurotoxicity in Autism Spectrum Disorders

Microglia represent the resident immune cell of the CNS.[61] Under normal conditions microglia exist throughout the brain in a resting state referred to as ramified microglia. Any insult to the brain can trigger rapid activation of ramified microglia, resulting in an ameboid morphology. Ameboid microglia can travel via the extracellular space, acting as scavengers of injured and dying cells. During embryogenesis, activated microglia remove pruned neurons and dendritic processes, thus playing a vital role in brain development.

In addition, microglia are antigen presenting cells (APCs) and thus possess a wide assortment of surface antigens and receptors including ß2-integrins, leucocyte common antigen (LTA), immunoglobulin Fcγ receptors, major histocompatibility complex class I (MCH-I) and MCH-II glycoproteins.[62]

Human microglia while in a ramified state constitutively express transcripts for mRNA of IL-1ß, IL-6, IL-8, IL-10, IL-12, IL-15 and TNF-α, as well as macrophage inflammatory protein-1 (MIP-1), MIP-1ß and MCP-1. When activated, microglia express both inflammatory cytokines (Il-1ß, IL-6, IL-8 and TNF-α), immunomodulatory cytokines (IL-5, IL-12 and IL-15) and anti-inflammatory cytokines (Il-10 and Il-13).

In addition, human microglia also contain receptors for each of these cytokines, which can be activated to increase the release of additional inflammatory cytokines. While we still do not understand fully the mechanism of microglial activation, the MAP kinase family of enzymes play an important role.[63] There is evidence that microglia can respond differently to environmental stimuli determined, in part, by which MAP kinase is activated. For example, the extracellular signal-regulated kinase (ERK) is most responsive to growth factors and phorbol esters, while c-jun N-terminal kinase/stress activated protein kinase (JNK and p38MAP kinase) are activated by stress signals, such as lipopolysacchride (LPS) stimulation.[64]

In addition to immune cytokines, chemokines and surface antigens, microglia also secrete a number of neurotoxic molecules, including reactive oxygen and nitrogen species (ROS/RNS), lipid peroxidation products (LPO), nitric oxide and two excitotoxins-glutamate and quinolinic acid.[65,66] Likewise, a number of these products can also drive microglial activation and further secretion of neurotoxic molecules. This includes glutamate, quinolinic acid, interferons, inflammatory cytokines, chemokines and ROS/RNS and LPO products. [67-71]

Using purified cultures of second trimester human fetal microglia, Lee and co-workers demonstrated LPS induced mRNA for IL-1ß, IL-6 and TNF-α.[72] IL-1ß was a powerful stimulus for microglial activation, which confirms previous studies showing IL-1ß to be a major regulator of microglial cytokine activation and cytokine secretion.[73] This emphasizes the connection between systemic IL-1ß activation by vaccinations and systemic illness and activation of brain microglia.

In sheep, which reach term at 150 days, TNF-α appears first on embryonic day 30 (E30) and IL-1ß at E35-45 in the cortical plate.[73] This and the previous study not only demonstrate that microglial function begins early in embryonic life, but that the cytokines play a critical role in neurodevelopment. The highest levels of TNF-α and IL-1ß were present in the subplate zone at the time of most intense synaptogenesis. Further evidence of the importance of cytokines in neurodevelopment arise from the observation of considerable fluctuations in cytokine levels during pregnancy.[74]

Not only do microglia clean up the debris during pruning but they also secrete a number of growth factors, which may guide neuronal migration, enhance survival and promote dendritic arborization.[75] Microglia are known to secrete a number of growth factors including nerve growth factor (NGF), neurotrophin 3 (NT3), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), hepatocyte growth factor, and plasminogen. Most are brain region specific.[76]

It has been proposed that there are three basic states of microglia: a neurotrophic state or resting state, a bifunctional state, during which both cytotoxic and neurotrophic molecules are released and a cytotoxic state during which only neurotoxic molecules are released.[77] Normally, the latter is tightly regulated. There is evidence that microglia play a significant role in neural migration and dendrite development.[78,79]

There is also evidence that excessive microglial activation can disrupt neurogenesis and neurodevelopment. [80-83] This appears to involve inflammatory cytokines as well as glutamate excess.[84,85] Elevated levels of inflammatory cytokines and glutamate have been demonstrated in a number of studies of autistic children and adults. [9-11] In most of the amino acid studies, serum or blood glutamate levels were measured.[86-88] Hamberger and co-workers found significantly elevated CSF glutamate levels in four patients having Rett syndrome, which is known to have autism-like neurological symptoms.[89]

One of the hallmarks of infantile form of autism is an overgrowth of brain, with asymmetrical enlargement of the amygdala and cellular abnormalities in the brain stem and cerebellum, hippocampus, frontal lobes and parietal lobes.[38, 90] Neuropathologically, classic autistic patients have a reduced number of Purkinje cells in their cerebellum[91] and abnormally dense packing of neurons in the amygdala and hippocampus.[92,93]

Despite these findings in classic autism, the increasing number of new cases appearing since the early 1980s, include a large number who do not show these dramatic changes in brain gross anatomy using scanning techniques. Yet, Courchesne has shown that scanning techniques may not be able to detect less obvious changes in the cerebellum.[94] The difference in severity appears to be the stage at which the insult arises. Postnatal injury is more likely to produce one of the lesser autism spectrum disorders rather than the more neurodevelopmentally severe classic autism.

In humans a considerable amount of postnatal brain development occurs, with the greatest period of synaptogenesis and pathway development occurring during the first two years after birth. Elevated glutamate and cytokine levels secondary to microglial activation would be expected to affect postnatal brain development as well, as evidenced by effects on adult neurogenesis. [95-97]

There exist considerable interaction between microglia, astrocytes and neurons. Resting microglia are known to secrete low levels of several cytokines and growth factors that play a role in neuronal maturation, neuroprotection, dendritic growth and stabilization.[98] When activated acutely, microglia normally secrete both neurotoxic factors and neuroprotective factors.[99] Growing evidence indicates that chronic activation of microglia and astrocytes produces a state of predominant neurotoxicity.[100-102] Activation of microglia can occur from a number of CNS insults and by the presence of cytokines themselves in an autocrine manner.

Of particular concern in the case of autism is the interaction of excitatory amino acid neurotransmitters and cytokines. A number of studies indicate that there is a co-stimulatory interaction between the two.[103-105] One method by which cytokines and excitotoxins interact was shown by Floden and co-workers in which they demonstrated TNF-α and glutamate synergistically stimulated neuronal inducible nitric oxide synthease (iNOS) expression, with subsequent peroxynitrite production, which led to neuronal death.[106] Stimulation with either TNF-α or glutamate alone produced no toxicity, but when added together produced robust neurotoxicity. Blocking either peroxynitrite or iNOS prevented the toxicity, indicating that peroxynitrite was the toxic factor. Nitration reactions with cellular components and molecules can impair neuronal function.

The NMDA receptors are co-localized with the TNF-α receptors TNFR1 and TNFR2 on the neuron, allowing cross-talk during stimulation. TNFR1 is predominately neurotoxic and TNFR2 is predominately neuroprotective.[107] Neurons contain primarily TNFR1 type receptors. As regards the co-localized NMDA receptors, it has been shown

that subpopulations of NMDA subunits determine the susceptibility of NMDA-dependent death and thus the eventual neurodevelopmental outcome.[108] Similarly, it has been suggested that regional brain susceptibility to immune/excitotoxic injury is dependent on microglial population densities.[109]

Recently, Takeuchi and co-workers demonstrated that TNF-α increases glutamate release from microglia by up-regulating glutaminase, the enzyme responsible for glutamate generation from glutamine.[110] In addition, they demonstrated that release of glutamate was not via glutamate transporters (EAATs) or the X_c^- heteroexchange system, but rather by the connexin 32 (Cx32) hemichannel of the gap junction mechanism.

One of the most comprehensive examinations of immune activation in the autistic brain was conducted recently by Vargas and co-workers.[48] In this study, they performed immunocytochemistry, cytokine protein arrays and enzyme-linked immunosorbant assays on the brains of 11 autistic patients dying from non-infectious causes. Their ages ranged from age 3 years to 45 years. In this study they examined brain tissue and CSF for both inflammatory and anti-inflammatory cytokines, chemokines and growth factors. Control patients were chosen in the same age ranges and with relatively similar causes of death.

They found the greatest immune-related damage in the cerebellum with extensive loss of Purkinje cells and granules cells in 9 of 10 cerebella examined. No significant histopathological changes were seen in the age-matched controls. Widespread microglial activation was seen throughout the brain with combined microglial/astrocyte activation most prominently seen in the cerebella, anterior cingulate gyrus and less so medial frontal gyrus.

No correlation was found between the degree of changes and age or clinical developmental regression. A mixed pattern of proinflammatory and anti-inflammatory cytokines and chemokines and growth factors were seen, with a predominance of proinflammatory activity. Similar finding were seen in the CSF. The main source of the cytokines was the astrocytes.

The most consistently elevated proinflammatory factor was macrophage chemoattractant protein-1 (MCP-1), which plays a major role in innate immune reactions and is a vital mediator for monocytes, macrophage and T-cell activation and trafficking into injured zones.[111] Also significantly elevated in both brain and CSF was IL-6, which not only plays a vital role in neurodevelopment, but, when chronically elevated, especially in the presence of excitotoxins, can disrupt brain development and function.[112,113]

Vargas et al noted that none of the autistic brains demonstrated leptomeningeal, parenchymal or perivascular inflammatory infiltration, suggesting that the systemic immune system was not playing a significant role in the pathological changes, but also noted that they were seeing the chronic phase and not the acute onset. It is known that with priming of brain microglia, repeated episodes of systemic immune activation can trigger a chronic, exaggerated brain immune response.[114]

There are several explanations for this in relation to systemic immune activation as a triggering mechanism. It may be that mercury and aluminum from vaccines as well as other sources, by accumulating in the brain, are acting as the innate trigger. It has been shown that both of these metals can trigger microglial activation and neurodegenerative effects.[115,116] By accumulating in the brain, they may act as chronic immune stimulants.[117,118] Studies have shown that mercury preferentially accumulates in

astrocytes.[119] It is also suspected that infiltrating bone marrow myeloid progenitor cells are converted into microglia during inflammatory brain conditions, which would be even more likely with chronic immune activation, thus explaining the lack of continued systemic immune activation.[120]

It is obvious that activated microglia, with subsequently activation of astrocytes, can result in an environment that is hostile to developing neurons and the development of dendritic connections and synapses. The timing of the injury during the developmental profile of the various neural systems allows for a complex array of final neurological events and neurological syndromes. The competency of the central nervous system's protective mechanisms, especially the antioxidant systems, DNA repair enzymes and other cellular mechanisms of protection, determines, to a large extent, the final outcome. The competency of the immune system is also a major determinative factor.

Systemic Immune Stimulation and Microglial Activation

A number of studies have reported worsening of neurological disorders with systemic infection, including Alzheimer's disease, multiple sclerosis, frail elderly and ALS.[121-124] Likewise, experimental studies have also shown microglial activation following systemic immune stimulation. For example, Vereker and co-workers demonstrated degenerative changes in the hippocampus and entorhinal neurons following intraperitoneal injection of lipopolysacchride (LPS) in rats.[125]

In one study it was shown that repeated intraperitoneal injection of LPS during a presymptomatic stage, shortened the lives of transgenic ALS mice.[126] In addition, peripheral stimulation of immunity with LPS can increase brain cytokine secretion and exaggerate sickness behavior as well as neurodegeneration.[127,128]

Churchill and co-workers demonstrated that systemic IL-1ß could increase brain IL-1ß mRNA and that the effect was blocked by vagotomy.[129] Systemic IL-1ß also increased TNF-α and IL-6 mRNA induction in the nucleus tractus solitarius, hypothalamus, hippocampus and somatosensory cortex, most of which are affected in the autistic brain. When TNF-α was increased systemically, brain mRNA for TNF-α and IL-1ß were increased in the hypothalamus, hippocampus and somatosensory cortex, but IL-10, a powerful anti-inflammatory cytokine, did not increase. Of particular interest was that IL-1ß increased IL-6, IL-1ß and TNF-α in the amygdala and decreased the growth factor BDNF in the same nuclei. Amygdalar involvement is thought to play a significant role in the social deficiencies of autistic children.[130]

It is also known that IL-1ß activates neurons in the central nucleus of the amygdala[131] and that this same nucleus plays a major role in hypothalamic-pituitary adrenal axis response to systemic immune stimulation.[132] Interestingly, some autistic children have been shown to have low corticoid secretion[133], while others have found hypersecretion of cortisol, thought to be stress related.[134] This could indicate abnormalities in the glutamatergic-controlled HPA triggered by inflammation and excitotoxicity.[135] Since normally, cortisol secretion via the HPA controls the immune response, preventing excessive immune-mediated damage, low levels would aggravate chronic microglial activation.

Systemic activation of brain microglia can take place through the BBB-vascular interface, choroid plexus and by way of vagal afferents. Likewise, any disruption of the

BBB will increase the likelihood of systemic interaction with brain microglia. During early development, even postnatal, the BBB is considered to be incompetent. Oxidative stress is also known to activate microglia and systemic LPS has been shown to induce oxidative stress in the brain.[136]

Another important process is priming of microglia. Preexisting microglial activation has been shown to magnify neurodegenerative disease with subsequent systemic immune stimulation.[114,137] Using ME7 prion protein stimulus, Cunningham and co-workers found that priming of microglia in the hippocampus in mice followed by systemic intraperitoneal challenge with LPS produced a 3-fold higher increase in brain IL-1ß than when microglia were not primed.[138] IL-1ß, being the central controlling cytokine for microglial activation, at these high levels, would explain the chronic activation seen in the Vargus study. TNF-α levels increased 1.7-fold higher than unprimed state and IL-6 3-fold greater. Interestingly, they found no difference in TGF-1ß secretion, a major immune modulator, with it being elevated equally in the primed and unprimed state. This, as in the Vargas et al study, demonstrates a predominance of an inflammatory cytokine profile.

In the autistic patient, this would be similar to one of several situations. It has been observed that autistic children have early, and repeated, systemic infections, usually middle ear infections. This would serve to prime the microglia. A subsequent vaccination or vaccinations would be expected to produce an exaggerated microglial reaction, based on the priming effect and each inoculation would prime the microglia for the next inoculation.

Another situation would be inoculating children with live vaccines, such as the MMR vaccine (measles, mumps and rubella). It has been shown that the measles virus is retained in the brain over a lifetime following early exposure.[139] Once the virus was established within the brain, subsequent MMR vaccines could trigger an exaggerated immune response in the primed microglia. Even the presence of non-viable viral components could act as activators of microglia.

In addition, retention of vaccine adjuvants in the brain, such as mercury and aluminum, would also act to prime brain microglia. Additional inoculations, especially if spaced close together, would be subject to this priming effect. It has been shown that vaccine adjuvants can cause prolonged activation of the systemic immune system.[140]

Taken together this evidence clearly indicates that systemic immune stimulation can activate the brain's microglia and that priming of the microglia can cause a magnification of the brain's immune response. It is also clear that repeated systemic immune activation further enhances the destructive nature of CNS immune activation, especially if prolonged. Since the developing child's immune system, including the microglia, are active during early development, excessive and repeated activation can significantly interfere with brain development and function, as discussed earlier.

The Gastrointestinal Source of Chronic immune Stimulation

That chronic inflammation is playing a significant role in autism is beyond dispute, the source or sources of chronic stimulation is less clearly defined. As we have seen, recurrent vaccination with powerful immune adjuvants can produce prolonged and intense activation of brain microglial immunity and neurodegeneration and that "primed" microglia exhibit exaggerated immune reactions. It has also been suggest that another

source of immune activation can be from food allergies[141,142] or chronic intestinal infections with Candida or other dysbiotic organisms.[143]

Wakefield and co-workers also suggested a relationship between severe intestinal inflammation secondary to MMR vaccines and autism.[144] They proposed that intestinal inflammation produced malabsorption. A more recent study by Ashwood and Wakefield found that peripheral blood lymphocytes as well as mucosal CD3+TNF-α and CD3+IFN-γ cytokine responses were significantly increased in children with autism spectrum disorder as compared to non-inflamed control children.[145] The critical difference between children with Crohn's disease and those with ASD was that in the latter, peripheral and mucosal IL-10 responses were markedly lower. This not only indicated a gastrointestinal autoimmune reaction in autistic children but a suppression of the cytokine known to regulate immune termination, IL-10.

Several studies have shown a cross reaction between food derived proteins and neuron specific antigens. Vojdani and co-workers examined nine different neuron-specific antigens and three cross reactive peptides, which included milk proteins and found that autistic children had the highest IgG, IgM, IgA antibody reaction against all nine neuron antigens as well as a cross-reaction to all three peptides.[146] In a follow-up study in which they assessed the reactivity of sera from 50 autistic patients as compared to 50 healthy controls, Vojdani et al demonstrated that a significant number of the autistic children expressed antibodies against gliadin and cerebellar Purkinje cells simultaneously.[147]

In a number of studies reactions to commonly found colon bacterial organisms are seen to occur.[146,148] Candida infections are commonly seen in children with autism spectrum disorders and may also act as a source of strong, chronic immunologic reactivity, especially if they penetrate the gut wall.[149,150] The presence of beta-1,6 glucan in the cell wall of the organism appears to be the most powerful immune component.

Black et al in a study of 96 autistic children compared to 449 healthy children found no greater incidence of gastrointestinal disease in the autistic children.[151] Their study included only obvious gastrointestinal disease and symptomatology and recognized that they might miss more subtle symptoms of GI disease.

Another disorder that has shown a strong connection between immunological reactivity to food-based peptides and neurological dysfunction is celiac disease.[152] In this disorder patients are sensitive to gluten-containing diets (wheat, barley and rye). Approximately 6% will present with neurological complications, most frequently cerebellar ataxia. Burk and co-workers not only found cerebellar ataxia and oculomotor findings but also subtle cognitive impairments and difficulty with the Wisconsin Card Sorting Test indicative of executive prefrontal deficits.[153] The MRI scan demonstrated atrophy of the cerebellum.

Of interest is that several researchers have noted a lack of gastrointestinal symptoms in a number of these patients. For example, Hadjivassilou and co-workers found gastrointestinal symptoms in only 13% of patients with MRI evidence of cerebellar atrophy and clinical findings of sporadic cerebellar ataxia.[154] The MRI changes were not limited to the cerebellum, since they also found white matter hyperdensities.

Sporadic ataxia is the most common form of ataxia and this study found that gluten sensitivity accounted for 41% of cases, making it the most common cause of ataxia. Hu et al examined 13 patients with celiac disease with neurologic involvement and found

cognitive impairment in all patients.[155] Slow, progressive neurological onset was characteristic, with development of acalculia, confusion, personality change and amnesia.

Two of the patients underwent brain biopsy and two came to autopsy. All demonstrated gliosis, indicating microglial activation. One patient demonstrated the findings of frontotemporal lobar degeneration with histological involvement of the frontal and temporal cortices and hippocampal dentate granular cell layer. Not all patients improved with gluten free diets, either due to poor compliance or progressive neurodegeneration, as seen with such immune-related neurological diseases as multiple sclerosis treated with immune suppressing drugs. It is suspected that progression is secondary to chronic excitotoxic neurodegeneration.[156]

Hadjivassilou and co-workers tested the sera from cases of gluten ataxia, patients with newly-diagnosed celiac disease without neurological involvement, patients with other cerebellar diseases and healthy controls using immunostaining with IgG antigliadin antibody on human cerebellar and rat CNS tissue.[157] They found that 12 of 13 of the cases of gluten ataxia stained the Purkinje cells intensely. Some of the cases of celiac disease without neurological symptoms stained the Purkinje cells mildly. No staining was seen in controls. This indicates that patients with gluten ataxia have antibodies against Purkinje cells.

Taken together, these studies clearly indicate that allergies to food components and colon microorganisms can activate CNS microglial innate immunity, resulting in a diverse array of neurological disorders and behavioral changes. They also indicate that obvious gastrointestinal disorders do not have to exist. With priming of the microglia, as would occur with recurrent infections or repeated vaccinations early in life, the intensity of the brain's immune response to food-based peptides would be drastically enhanced and act as a continuous source of brain immune stimulation.

Excessive Androgens and Autism

There is strong evidence that mercury exposure in humans increases androgen levels. For example, Barregard and co-workers reported that there was a significant correlation between increasing concentration of mercury in chloralkali workers and testosterone levels.[158] Animal studies also show a link between sex steroid production and mercury dosing.[159] Studies have also shown a link between elevated prenatal testosterone[160], postnatal serum testosterone[161] and autism spectrum disorders.

As to the mechanism of testosterone elevation by mercury exposure, it has been suggested that Hg^{2+} directly causes a defect in adrenal steroid biosynthesis by inhibiting the activity of 21 alpha-hydroxylase[162], while others have suggested inactivation of hydroxysteroid steroid sulfotransferase either directly[5] or by way of inflammation.[163] It has also been shown that DHEA-S, the proposed storage form of active DHEA, is also significantly lowered in autistic disorders.[164]

Kim et al have shown that even very small doses of LPS (1nM) can dramatically decrease the levels of mRNA for Sult2A1 and PAPSS2, which are responsible for sulfonation of a number of endogenous hydroxysteroids, bile acid and xenobiotics as well as sulfonation of DHEA to DHEA-S.[165] Normally DHEA-S plasma levels are 300 to 500-fold higher than DHEA levels. They found that TNF-α and IL-1ß was responsible for the decrease. Unlike autistic patients, DHEA levels were not increased, which may be caused

by mercury toxicity. Reductions in DHEA-S are common with other chronic inflammatory disorders, such as rheumatoid arthritis.[166]

In keeping with the finding of a defect in transsulfuration, one frequently sees associated elevations in androgens and elevations in homocysteine. For instance, several workers have found elevated levels of homocysteine in cases of polycystic ovary syndrome.[167,168] Normally, men have higher homocysteine levels than women, thought to be secondary to higher androgen levels.[169]

Androgen excess interferes with the conversion of homocysteine to cysthathionine, which by conversion to cysteine becomes a major source of glutathione.[170] Thus androgen excess can not only raise homocysteine levels, it can lower glutathione, a major antioxidant in brain. Other pathways in the methionine cycle are also affected, which may partially explain the significant reduction in methionine seen in autistic children, as well as s-adenosylmethionine levels.[171,172]

James and co-workers found not only low total glutathione levels in autistic subjects, but also oxidized glutathione levels that were 2-fold higher, which strongly indicates oxidative stress.[171] Several of the enzymes utilized in the methionine cycle, such as methionine synthase, betaine homocysteine methyltransferase and methionine adenosyltransferases, are known to be redox-sensitive enzymes.[173,174] With the chronic elevation of ROS, RNS and lipid peroxidation products in the autistic brain, one would not be surprised at suppression of these enzymes.

Vitamin B-12 and folate interplay in generating methyl groups during the methionine cycle. A recent study found an increased frequency in mutations of the C677T allele of methylenetetrahydrofolate reductase enzyme in autistic children.[175] The same genetic mutation causes elevations in homocysteine.[176] In addition, studies have shown abnormal absorption of vitamin B12 from the ileum of autistic children.[177]

It is accepted that there is a dimorphic influence of sex steroids on both external male/female morphology as well as brain structure and behavior.[178] In addition, it has been suggested that autism represents a form of "extreme male brain", with normal male behaviors, such as a reduced ability to read nonverbal skills, different language skills and low theory of mind function, being accentuated.[179,180]

Support for this theory arises from studies of children with congenital adrenal hyperplasia (CAH), which is characterized by high levels of circulating androgens in both afflicted males and females. For example, in one such study, Knickmeyer et al, found that females affected with high androgen levels scored higher on the Autism Spectrum Quotient test than normal females.[181]

While this is suggestive of a link, despite high levels of testosterone in children with congenital adrenal hyperplasia, few are fully autistic, even though they may share some behavioral symptoms. In addition, many have other metabolic disorders that could contribute to symptomatology, such as electrolyte disorders.

This is not to say that these studies on CAD didn't show behavior effects, it's just that the serious defects in social cognitive function seen with autism are not observed. This indicates that more is involved with autism than elevated androgen levels early in development. For example, elevated androgen levels do not explain the chronic extensive immune activation seen in the autistic brain or the prolonged, widespread activation of microglia and astrocytes. It also doesn't completely explain the extensive

neuropathological findings and abnormal pathway development found in the autistic brain.

A number of studies have shown abnormalities in both morphology and function in the amygdala and prefrontal cortex of autistic children, something not accounted for with androgen excess alone.[182-184] Estrada el al have shown that supraphysiologic levels of testosterone (micromolar ranges) can initiate apoptosis of neuronal cells in culture, which should affect neural development.[185] Likewise, Geier and Geier found rather dramatic and rapid improvement in 11 consecutively treated autistic children using both mercury chelation and leuprolide acetate, a drug which lowers androgen levels. The children experience a two-fold drop in serum testosterone levels over three months. Improvements were seen in sociability, cognitive awareness and aggressive behavior, due mostly to lowered androgen levels, since the effects of mercury chelation usually take longer to manifest.

It should be noted that children fasting for blood test have been noted to show similar rapid improvements in behavior. The combination of elevated androgens, reduced glutathione protection against oxidative stress and elevated levels of homocysteine would be of considerable concern during brain development.

The Role of Androgens and Estrogens on Microglial Activation and Excitotoxicity

The question to be answered is by what mechanism does androgen excess affect neurodevelopment and neurologic function? There are several possibilities, yet they may be interrelated.

It is known that both testosterone and estrogen, at basal levels, are neuroprotective and play a significant role in neuronal development, migration, dendritic outgrowth and synaptogenesis.[186,187] Central to the effect of androgen excess appears to be generation of calcium oscillations by androgens, which have been shown to regulate not only neurite outgrowth, but also neuron migration.[188] These oscillation of calcium are not caused by stimulation of gene androgen receptors, but rather rapidly acting cell membrane G-protein-regulated receptors which activate endoplasmic reticulum calcium release by inositol 1,4,5 triphosphate and diacylglycerol signal transduction.[189] It was also shown that the calcium oscillations were not secondary to conversion of testosterone to estrogens by brain aromatase. These oscillations of intracellular calcium also code for cell differentiation in the CNS.[190]

The recent finding by Balthazart and co-workers that the glutamatergic system, primarily acting through the AMPA/kainate receptors, rapidly inhibits brain aromatase activity demonstrates another mechanism by which brain testosterone levels remain elevated in the autistic child.[191] Brain aromatase converts testosterone into 17ß-estradiol as an inducible enzyme.[192]

Studies have shown that both NMDA receptors and androgen receptors play a role in neuronal differentiation, migration and dendritic outgrowth by regulating calcium oscillations.[193-195] Calcium waves have also been shown to regulate growth cone function.[196] Of particular interest was the finding by Estrada and co-workers that low concentrations of testosterone induced calcium oscillations, but high concentrations produced sustained dose/dependent elevations in intraneuronal calcium levels, something

that would be expected to produce abnormal neuronal migration and neurotoxicity.[197] In their study they indeed found that higher doses of testosterone triggered apoptosis human neuroblastoma cells. The effect was dose dependent, with 1uM inducing significant cell death and 10uM being significantly more lethal. It is also of note that recent finding of region specific 5α-reductase, which converts testosterone to the more potent dihydrotestosterone, can result in specific regions of the CNS having testosterone levels higher than plasma levels.[198]

Others have noticed that there is a sex difference in terms of the outcome of neurological injury, with females making better neurological recoveries than males.[199, 200] Experimentally, Hawk and co-workers found that chronic testosterone replacement increased stroke damage and 17ß-estradiol treatment deceased damage in castrated male rats.[201] This is in keeping with the demonstrated protective effects of estrogens on brain, at least when in physiological ranges.

While androgen receptors have been demonstrated in the hypothalamus, hippocampus, preoptic area, amygdala and medial hypothalamus, they have also been demonstrated throughout frontal lobe areas as well and influence frontal lobe $GABA_A$ receptor regulation.[202-207] This demonstrates a more expanded behavioral effect of androgens than merely reproductive behavioral effects.

In another study, Yang and co-workers using both a murine hippocampal culture and an *in vivo* study using Sprague-Dawley rats, found that 10 uM of testosterone *in vitro* significantly increased glutamate toxicity.[208] Likewise, 10 uM of estradiol significantly ameliorated glutamate toxicity. In the *in vivo* study, they used an implanted testosterone pellet for slow release of the hormone to minimize the stress of repeated injections. Using a middle cerebral artery stroke model, they found that the testosterone-implanted animals had a significantly larger volume of stroke damage than did controls.

Androgens, like excitotoxins, have been shown to enhance the inflammatory mediator NFkB and thereby increase COX-2 and iNOS activation, leading to free radical generation, lipid peroxidation and increased secretion of glutamate from microglia.[209,210] Using both an excitotoxic and stab wound injury to hippocampus, Garcia-Overiero and co-workers demonstrated that both lesions could induce androgen and estrogen receptors on glia.[210] Estrogen receptor alpha (ERalpha) was expressed on astrocytes and androgen receptors (AR) were expressed on microglial membranes.

Both receptors were observed to appear 3 days after the injury, with the maximum of ERalpha and AR immunoreacting glia appearing at day seven, and returning to baseline at 28 days. Taken together, these studies indicate that chronic elevation of testosterone activates microglia, triggering the release of a number of neurotoxic elements including the excitotoxins glutamate and quniolinic acid. Indeed, DonCarlos and co-workers have shown that of the glial cells only activated microglia express androgen receptors, whereas activated astrocytes express estrogen receptors.[211] They also found that AR immunostaining was heavier in frontal cortex than the hypothalamic-limbic structures. In addition, the demonstration that microglia direct neuronal precursor cell migration and differentiation and that activated microglia can increase neuronal numbers significantly, may explain the hypercellularity seen in certain areas of the autistic brain, particularly the amygdala.[78]

When androgen levels are chronically elevated, microglial activation would not only be enhanced but toxicity of secreted glutamate and inflammatory cytokines would be

exaggerated. Unlike the adult brain, this combination of inflammatory cytokines, androgens and excitotory neurotransmitters would not only precipitate chronic neurodegeneration, but also alter progenitor cell differentiation and maturation, dendrite outgrowth and arborization, synaptic development and stabilization and neuronal migration.

Homocysteine, Excitotoxicity and the Developing Central Nervous System

Homocysteine, which is elevated in many autistic children, is involved in various transsufuration reactions, such as cysteine synthesis, re-methylation for methionine synthesis and trans-methylation of DNA, proteins, lipids and the biosynthesis of neurotransmitters and some hormones. While cysteine itself is known to be a powerful excitotoxin[212], especially in an alkaline environment, in the autistic low cysteine levels are seen.[213]

Elevated homocysteine, even to moderate levels, is associated with Alzheimer's disease[214], age-related memory loss[215], schizophrenia[216], neural tube defects[217], seizures[218] and neurobehavioral toxicity of chemotherapeutic agents[219]. Homocysteine oxidizes to a number of L-glutamate analogues (L-homocysteine sulfinic acid (L-HCSA) and L-homocysteic acid (L-HCA)] and L-aspartate analogues (L-cysteine sulfinic acid (L-CSA) and L-cysteic acid (L-CA)] with significantly greater excitotoxic effects than homocysteine itself.[220]

Recent studies have shown that oxidized homocysteine metabolites activate NMDA receptors as well as metabotropic receptors and that in cerebellar granule cells neurotoxicity involves a co-stimulation of NMDA receptors and Group I metabotropic receptors.[221] Others have confirmed potent stimulation of excitatory metabotropic glutamate receptors by homocysteine metabolites.[222,223]

Lockhart et al found that hippocampal neurons were especially sensitive to excitotoxicity induced by the homocysteine oxidative product, L-homocysteic acid.[224] There is growing evidence that L-homocysteic acid, may be a glial transmitter, acting through astrocytic NMDA receptors.[225] With the metabotropic receptors of group I , as well as NMDA receptors being activated by homocysteic acid and homocysteine sulfinic acid, when in combination with high levels of extraneuronal glutamate, one sees a powerful amplification of the excitotoxic cascade.

There is also evidence that Purkinje cells have unique receptor properties in that they have few NMDA receptors and greater expression of non-NMDA receptors.[226] Homocysteic acid has been shown, as a excitotoxin, to act through NMDA receptors in hippocampal neurons and via non-NMDA receptors in Purkinje cells. With proinflammatory cytokines, ROS/RNS, lipid peroxidation products and mitochondrial depression-caused amplification of excitotoxicity, one can better understand the widespread loss of Purkinje cells seen in the cerebella of autistic cases. In essence, this is less of a direct autoimmune injury and more characteristic of bystander injury described by McGeer and McGeer as autotoxicity.[227]

Because both inotropic and metabotropic glutamate receptors, as well as androgens, are acting through excess intracellular calcium accumulation, one can readily understand the critical role played by each in the process, as explained in the next section. Homocysteine oxidation products, such as homocysteic acid, homocysteine sulfinic acid

and cysteic acid, along with glutamate, inflammatory cytokines, chemokines and inflammatory prostaglandins trigger the autotoxic injury to a widespread area surrounding the immune reaction thus explaining the autopsy picture seen in the autistic brain.

The Role of Mercury in Autism

Both mercury and aluminum are considered neurotoxic metals, with mercury being significantly more toxic. Autistic children are exposed to a number of sources of mercury and aluminum. Mercury exposure can be from atmospheric sources, dental amalgam, fish consumption, exposure to certain pesticides and herbicides and vaccines. In most cases, children are exposed a number of such sources. Of particular concern to the child's developing brain is *in utero* exposure to mercury from the mother's dental amalgam, seafood consumption or vaccinations during pregnancy or immediately before conception. Because of the human brain's extensive postnatal development, mercury exposure after birth is also of major concern. Mercury has been shown to pass through the placental barriers rather easily, thus entering the fetus' circulatory system, and hence, brain.[228,229] The leading sources of aluminum are food and vaccines.

A number of studies have shown architechtonic abnormalities in the fetus following maternal exposure to mercury.[230-233] This can result in abnormalities in neuronal and glial proliferation, neuronal migration and the final cytoarchitecture of the brain, especially the cerebellum.

There is also evidence that ionic mercury is the most toxic form of mercury within the CNS, and that organic mercury is slowly demethylated in the brain to form ionic mercury, which can then be redistributed over time. Vahter and co-workers, for example, studied demethylation of methylmercury in Macaca fascicularis monkeys after oral dosing with 50ug/kg of methylmercury for 6, 12 or 18 months and found that the concentration of inorganic mercury slowly increased in all brain sites, but especially in the thalamus and pituitary.[234]

Recent studies have shown that there are toxicological and pharmacokinetic differences between methylmercury from seafood and ethylmercury from the vaccine preservative thimerosal. For example, Burbacher and co-workers, using monkeys exposed either to methylmercury (MeHg) or vaccines with thimerosal at birth and then at 1,2 and 3 weeks of age, found a significant difference in the blood half-life, with thimerosal's initial and terminal half-life being 2.1 and 8.6 days respectively and MeHg being 21.5 days.[235] They also found that ethylmercury's brain concentration was 3-fold lower than MeHg. Yet, of significant importance was the finding that 34% of ethylmercury was converted to ionic mercury in the monkeys' brains vs 7% for MeHg. Ionic mercury, besides being more toxic, is much more difficult to remove from the CNS, even with chelation.

Two studies measured the mercury burden of children receiving the recommended childhood vaccines. Redwood and co-workers found that at birth an infant received 12.5 ug of mercury, 62.5 ug at 2-months, 50 ug at 4 months, 62.5 ug at 6 months and 50 ug at 18 months, for a total mercury burden of 237.5 ug of ethylmercury during the first 18 months of life, which exceed the environmental protection agency safety guidelines for an adult.[236] In the second study, similar infant mercury exposures were seen.[237]

Effect of Mercury on Neurons, Microglia and Astrocytes

One of the most obvious toxic effects of mercury is the generation of abundant free radicals and lipid peroxidation products and antioxidants provide considerable protection against mercury-induced neurotoxicity.[238] Yet, a more complicated process appears to be involved in the generation of these free radicals, since blocking the NMDA glutamate receptor also significantly attenuates MeHg toxicity and reduces ROS generation as well.[239,240] It has also been shown that free radicals dramatically increase the toxic sensitivity of immature neurons to MeHg, so that previously non-toxic concentrations of MeHg became fully toxic[241], just as in the case of excitotoxins.[242]

One of the most involved free radicals in both mercury neurotoxicity and excitotoxicity is peroxynitrite, formed by an interaction between nitric oxide (NO) and superoxide.[241,243] Peroxynitrite is known to especially target the mitochondria, which reduces energy production and enhances ROS formation.[244] In addition, peroxynitrite, as a reactive nitrogen species, reacts with cellular proteins, particularly L-tyrosine residues, producing nitrotyrosine accumulation.

New evidence points to a strong connection between inflammation in the brain, mitochondrial failure and excitotoxicity through calcium activated inducible nitric oxide synthetase (iNOS) and formation of peroxynitrite.[245] Activated microglia are known to up-regulate iNOS and generate large amounts of peroxynitrite, which in turn not only triggers excitotoxicity but reduces cellular energy levels.[246,247] Reduction in cellular energy enhances excitotoxicity to the degree that even physiological concentrations of extracellular glutamate can be excitotoxic.[248] Recent studies have shown that mitochondrial dysfunction is commonly found in neurodegenerative diseases.[249,250] Also of note, studies have shown the mitochondria to have the highest intracellular levels of mercury on exposure to ionic mercury.[251]

One of the major functions of mitochondria, besides energy production, is calcium buffering. During excitotoxicity, much of the cytosolic calcium is removed by either the smooth endoplasmic reticulum (SER) or mitochondria and dysfunction of either can result in exacerbation of intracellular signaling, with resulting free radical generation, lipid peroxidation and activation of cellular death signals. Mercury, by disrupting cellular calcium channels and activating SER calcium signaling, further exacerbates the problem, leading to abnormal neurogenesis and neurodegeneration as well as microglial activation as described previously.[252]

Systemic stimulation of immunity utilizing LPS, increases brain oxidative stress, thus increasing sensitivity to excitotoxins and mercury.[253] In addition, as we have seen, systemic inoculation with LPS also increases brain microglial activation, inflammatory cytokine activation and enhancement of excitotoxicity. Likewise, these events are characterized by disruptions of calcium homeostasis, mitochondrial dysfunction and cellular energy loss, again, all events that have been shown to disrupt neurogenesis and induce neurodegeneration. The effect of overstimulation of glutamate receptors, particularly NMDA and AMPA receptors, is further enhanced by ROS, lipid peroxidation products and inflammatory cytokines, especially TNF-α.[254,255] Aluminum, like mercury, is a powerful inducer of brain ROS and LPO production.[116,117] Measures of oxidative

stress and lipid peroxidation have shown significant elevations in children with autism.[256,257]

It should also be noted that high levels of DHEA interfere with mitochondrial energy production, and as we have seen, DHEA levels are increased as much as 2-fold in some studies of children with autism spectrum disorders. [258] In this study it was found that high levels of DHEA suppressed complex I (NADH quinone oxidoreductase) in primary cultures of cerebellar granule cells without affecting other mitochondrial electron transport enzymes. In the in vivo part of their study, adult male mice were fed a diet containing 0.6% DHEA for 10 weeks followed by a normal diet to exclude acute effects of DHEA. They found that the neuron density was significantly lower in the primary motor cortex and hippocampus. They also noted that under hypoglycemic conditions the toxic effect of DHEA was significantly more pronounced. Because of the effects of complex I inhibition on neurogenesis, one would expect a different histological picture in immature or fetal mice. With DHEA levels being significantly elevated in autism spectrum disorders, it is reasonable to assume depression of mitochondrial function would occur, especially in the presence of other mitochondrial depressing factors such as elevated levels of peroxynitrite and mercury toxicity.[164]

Charleston and co-workers in their study of long-term exposure of monkeys to methylmercury described extensive microglial, as well as astrocytic activation throughout the brain as described in the brains of autistics by Vargas et al.[48,115] Of special importance, they found continued microglial activation in the group of monkeys in which MeHg exposure was stopped for 6 months, demonstrating that microglial activation persist long after exposure. It should also be noted that with priming by mercury-induced activation of microglia, further immune activation from any cause, vaccinations, systemic infections, food allergies, etc, would be expected to exaggerate brain excitotoxicity and inflammation.

While, astrocytes are the major source of glutamate, as well as critical inflammatory cytokines, microglia act as the primary mechanism of astrocyte activation and they can also secrete excitotoxic levels of glutamate upon stimulation.[61,259] This is especially so under conditions of mitochondrial dysfunction, magnesium deficiency and hypoxia/ischemia.

With astrocytes acting as the sink for mercury, concentrations reach significantly higher, neurotoxic levels in this cell type. Astrocytes also act as the primary site for glutamate uptake. A large number of studies have shown that glutamate uptake can be significantly altered by extracellular toxins, including TNF-α, ROS, RNS, lipid peroxidation products, and that uptake is sensitive to even small concentrations of mercury.[260-264] In fact, Brookes demonstrated that concentrations of mercuric chloride as low as 0.5 ug inhibited glutamate transport into astrocytes by 50%, and that no other metal tested, Al^{2+}, Pb^{2+}, Co^{2+}, Co^{2+}, Sr^{2+}, Cd^{2+} or Zn^{2+}, inhibited glutamate transport.[265] At this concentration, mercury is considered not to be directly cytotoxic.

Glutamate uptake is not the only neurotransmitter affected. Dave and co-workers found that methylmercury not only inhibited glutamate uptake in primary astrocyte cultures, but that it also inhibited Na+-dependent and fluoxetine-sensitive [^{3}H] 5-HT uptake as well.[266] This could, in part explain the elevated serotonin levels seen in autism.[267] Of concern with chronically elevated levels of serotonin is the fact that one of

its metabolic products, quinolinic acid is also an excitotoxin secreted from activated microglia.[268]

Effect of Mercury on Glutamate Transporters

Glutamate regulation occurs through four primary mechanisms: the X_{AC}^- transporters (excitatory amino acid transproters-EAAT1-5), the X_c^- cystine/glutamate antiporter, conversion of glutamate into glutamine by glutamine synthetase and metabolic diversion into Kreb's cycle. Inhibition of the EAAT glutamate transporters may be primarily through oxidation, since antioxidants can reverse the inhibition.[269,270] The transporters contain sulphydryl groups, which would make them vulnerable to mercury as well as oxidation.[271] It is also known that the transporters are dependent on protein kinase C and that mercury inhibits its function.[272,273] One of the mechanisms for estrogen protection against excitotoxicity is its ability to enhance glutamate transport into the astrocyte.[274]

Not only do the glutamate transporters play a vital role in preventing excitotoxicity, they also play a major role in brain development, as there is a programmed rise and fall in the different transporters during brain development.[275] In one study, Klugler and Schleyer found that the glutamate transporter GLAST (EAAT1) was expressed in higher levels earlier in development than GLT-1 (EAAT2) in the rat hippocampus and that both the glutamate transporters and glutamate dehydrogenase were increased at birth and rose to adult levels between P20 and P30, indicating an important control system over glutamate levels during postnatal development.[276] Mercury has also been shown to suppress glutamate dehydrogenase activity as well.[277]

It has also been shown that Purkinje cells are very dependent on GLAST and EAAT4 for resistance against excitotoxicity induced by hypoxia/ischemia.[278] GLAST is expressed in Bergmann glia and EAAT4 in the perisynaptic region of Purkinje cell spines.[279] This could also explain the dramatic loss of Purknje cells in autism, since mercury toxicity alone usually spares the Purkinje cells and targets cerebellar granule cells.[280] A combination of inflammatory bystander injury, ROS-RNS/LPO accumulation, androgen excess and excitotoxicity dramatically increase the damage, mainly because of hyperexcitability of NMDA and AMPA receptors and chronic microglial activation, with release of neurotoxic elements.

Juarez and co-workers demonstrated a dramatic increase in extracellular glutamate following methylmercury instillation in the frontal cortex of 15 freely moving awake rats using a microdialysis probe.[281] They found a 9.8 fold rise in extracellular glutamate following a MeHg dose of 10 uM and 2.4-fold rise using a 100 uM dose. It is known that a dose of 10 uM of MeHg produces a 50% inhibition of glutamate uptake into astrocytes.[282] Brain trauma in rats has been shown to produce a 2.8-fold rise in extracellular glutamate.[283]

Mercury is also known to be a potent inhibitor of glutamine synthetase activity, which when inhibited, causes a buildup of extracellular glutamate.[284] This can lead to excitotoxicity and an alteration in neuronal migration and progenitor cell differentiation.

Mercury's Effect on Glutathione, Metallothionein, Excitotoxicity and Autism

Another frequent finding in autism is a lower glutathione levels, which is also common with mercury toxicity and excitotoxicity.[285-287] As one of the principal intracellular antioxidants, glutathione scavenges a number of reactive oxygen and nitrogen species, including peroxynitrite. It has also been shown to have a neurotransmitter function, binding to its own synaptic receptors and in addition, has been shown to modulate glutamatergic excitatory neurotransmission by displacing glutamate from ionic receptors.[288,289] At high extracellular concentrations glutathione enhances NMDA receptor activity, increasing the risk of excitotoxicity.[288]

Astrocytes are the sole source of glutathione for neurons, making it particularly susceptible to mercury inactivation, since astrocytes are also the principle site of mercury accumulation in the CNS.[290] Mercury has been shown to lower glutathione levels in embryonic neuronal cells as well as adult neurons.[291,292] Low glutathione levels have been associated with a number of neurodegenerative conditions, especially Parkinson's disease, as an early event.[293-295]

Glutathione production by astrocytes is dependent on the sodium-independent X_c^- cystine/glutamate antiporter, which exchanges intracellular glutamate for extracellular cystine utilized by the astrocyte to produce glutathione.[296] High levels of glutamate inhibit cystine entry into astrocytes, resulting in low glutathione levels, as we would expect with the elevated glutamate levels seen in autistics and those exposed to mercury.[297]

Another protective system impacted by mercury is metallothionein. Risling and co-workers have shown that exposure of rat neonatal primary astrocytes to methylmercury constitutively increase the production of metallothionein-1 (MT-1) and MT-2.[298] Aschner and co-workers demonstrated a 14-fold increase in MT-1 mRNA upregulation in full term fetal rats exposed in utero to elemental mercury vapor.[299]

Beside their role in heavy metal detoxification, metallothioneins function to control inflammation, oxidative stress and promote brain repair.[300] They have also been found to play a significant role in protection against excitotoxicity.[301] MT-1 and MT-2 play the most significant role in protection against neuroinflammation and have been shown to reduce the number of activated microglia during injury.[302] With a significant number of metallothionein molecules bound with mercury, they would be less able to carry out their anti-inflammatory and antioxidant functions.

There is abundant evidence that mercury, particularly in its ionic form, is toxic to neurons and less so glial cells, and that organic forms of mercury are demethylated slowly to form ionic mercury, with accompanying redistribution in the CNS. Because of mercury's effects on a number of enzymes, mitochondrial function, gene function, microglial activation, inflammatory cytokine release, antioxidant systems and glutamate metabolism, it becomes a major player in abnormal brain development as well as neurodegenerative-associated excitotoxicity. Most of these effects occur at very low micromolar or submicromolar concentrations.

Because few studies have looked at total accumulated concentrations from multiple sources, such as atmospheric mercury, seafood sources, thimerosal-containing vaccines and dental amalgam, the impact of mercury has been grossly underestimated by many experts in autism spectrum disorders.

Food Additives and Other Neurotoxins

Also of concern are the diets of autistic children, since many commercial foods contain substances and additives that have been shown to be neurotoxic, such as glutamate, aspartate, aluminum and fluoride. Of particular concern are glutamate and aspartate additives. Orally ingested glutamate, as MSG, has been shown to significantly raise blood levels of glutamate in animals and humans. Blood levels in humans can increase from 20 to 45-fold[303] and even higher in certain neurodegenerative diseases.[304]

It has also been shown that glutamate from ingested or injected MSG can pass through the placenta and accumulate in the fetal brain.[305] In fact, the glutamate level was 2-fold higher in the fetal brain than the maternal brain level in this study. A great number of studies have shown that elevated levels of glutamate in the blood can produce numerous lesions in the brain particularly in the hypothalamic, hippocampal and entorhinal areas, and can disrupt neuroendocrine function.[306-309]

Olney and co-workers have shown that the immature brain is approximately 4x more sensitive to glutamate excitotoxicity as is the adult brain.[310] Hypoglycemia, hypoxia and ischemia, which commonly occur during development, greatly aggravate excitotoxicity.[311] And, as shown, inflammatory cytokines, in particular IL-1ß and TNF-α, also magnify excitotoxicity.

Recent studies have shown that feeding of MSG early in life can lead to prolonged free radical and lipid peroxidation generation in the brain that can last until adulthood.[312,313] Since MSG and other forms of free glutamate and asparate are frequently used as food additives, this would put the child's brain at risk throughout most of the period of rapid brain growth and maturation, which extends during the first two years of life. Many foods, including toddler foods, contain several forms of glutamate additives, such as hydrolyzed protein and sodium or calcium caseinate in concentrations known to produce brain lesions in the rat and mouse.[314] Humans are 5x more sensitive to the excitotoxic effects of oral MSG than are mice and 20X more sensitive than monkeys.[303]

In addition, exogenously applied glutamate has been shown to prime microglia and in previously primed microglia, elevated brain levels of glutamate would trigger intense inflammatory cytokine and chemokine release as well as the release of excitotoxins from both microglia and astrocytes.[315]

A number of studies have shown that MSG can alter behavior and learning when given to animals postnatally.[43,317] For example, Frieder and Grimm demonstrated that oral feeding of MSG to pregnant rats produced profound defects in learning that only affected the males.[319] In a follow-up study they demonstrated a 25% fall in brain ChAT activity during early development and that norepinephrine was reduced to 25% of normal levels in the frontal lobes.[320] These neurotransmitter levels were not altered in the hippocampus.

Sanabria and co-workers demonstrated a dramatic decrement of long-term potentiation (LTP) field excitatory postsynaptic potential in adult rats at 60 days, after being exposed to MSG during the first 10 days of postnatal life.[321] The animals also failed to maintain or consolidate LTP, indicating a chronic impairment of CA1 synaptic plasticity, a major memory mechanism. Examination of the animal's hippocampus demonstrated decreased neuron numbers and abnormalities of dendrite arborizations and spine density in the CA1 zone as compared to controls. In addition, extensive microglial

activation was present. This confirms other reports showing long-term damage after neonatal exposure to MSG.[319,320,322]

Damage following neonatal exposure to MSG is not limited to the hippocampus. Gonzales-Burgos and co-workers exposed male rats to 4mg/g MSG subcutaneously at PND 1,3,5 and 7 and found fewer neurons and shorter, less ramified dendritic processes in the 3rd layer pyramidal neurons of the prefrontal cortex.[323] The cerebellar cortex and Purkinje cells are also sensitive to systemically administered MSG.[324]

Using radiolabeled [^{3}H] glutamate, Yu et al demonstrated penetration of the placental barrier by glutamate and its distribution in the fetal brain.[305] They also found that MSG given to maternal mice (2.5mg/g or 4mg/g) during later pregnancy decreased the threshold for seizures at 10 days postnatal and significantly impaired Y-maze discrimination learning in their offspring when studied at day 60. Importantly, these behavioral changes occurred without damage being detected by light microscope in the hypothalamus or periventricular organs, areas very sensitive to MSG neurotoxicity. This indicates that significant impairment of learning and behavior can occur at doses of MSG below that which produces obvious neuron death.

Administering MSG systemically to animals has been shown to increase oxidative stress and lipid peroxidation in peripheral tissues and brain as mentioned.[312,313] Giving MSG on post-natal day (PND) 1-10 has been shown to increased lipid peroxidation and alter antioxidant defenses in the midbrain and frontal cortex of rats lasting as long as PND 90.[313] In this study, lipid peroxidation was increased 56% in these brain areas. These studies indicate that exposure to dietary MSG during neonatal periods can produce very prolonged ROS/RNS and lipid peroxidation generation, aggravating any preexisting oxidative stress. Studies have also shown advanced oxidative stress and lipid peroxidation in autism.[325-327]

In addition, one sees elevations in SOD activity and decreased catalase activity in autism.[325] Reduced catalase in the presence of normal or elevated glutathione peroxidase activity can increase free radical damage because of the elevation in hydrogen peroxide, which subsequently breaks down into hydroxyl radials. Another biochemical change commonly found in autistic cases is low reduced glutathione levels.[328] Exposure to MSG is also known to lower reduced glutathione levels.[329,330]

NMDA receptors are known to be fully functional in early rat embryogenesis. Beas-Zarate and co-workers demonstrated, using reverse PCR methods, that gene expression for NMDA receptor subunits NR1, NR2A and NR2B in the striatum and hippocampus increased in rats exposed neonatally to MSG.[331] In cerebral cortex the NR2B subunit was increased, demonstrating that sensitivity to glutamate can be induced by the actions of glutamate on specific genes controlling subunit expression. They also found extensive microglial activation with MSG exposure.

Studies by Mitani and co-workers have shown that NMDA neurotoxicity was most prominent at PND 15 and not at birth.[332] This may be due to an increase in the NR2C NMDA glutamate receptor subunit expression at a later time postnatally, which is insensitive to magnesium blockade of voltage gated receptors. Ironically, this follows the increasing toxicity of mercury in the cerebral cortex and hippocampus of rats, which peaks at PND 14 and in the cerebellum at PND 35.[333]

Babies and small children are exposed to concentrations of MSG, hydrolyzed proteins, soy protein isolates, aspartame and occasionally cysteine that have been shown

to be excitotoxic in experimental studies.[334] Xu and co-workers found that MSG given i.p increased both brain and intestinal TNF-α levels 3-fold.[335] Inflammation in both tissues increased as a result of MSG-induced NFkB activation. The presence of a brain insult (ischemia) produced an even greater elevation in brain TNF-α levels in the MSG treated animals, again emphasizing the importance of priming.

Both clinical and subclinical seizures are known to occur in relatively high rates in autism spectrum disorders.[336,337] Likewise, a number of studies have also shown that MSG given systemically can lower seizure threshold and prolong seizures, with the immature brain being significantly more susceptible.[338-341]

Related to the child's brain and excitotoxic induced seizures, as well as neurodegeneration, is the finding of reduced GABA function. Even though during early embryogeneis GABAnergic neurons are excitatory, with maturation they play a predominantly inhibitory role.[342,343] It is known that GABA neurons possess metabotropic receptors, which means they are regulated by glutamate-type receptors.[344] A recent study found that some autistic children have defects in glutamic acid decarboxylase (GAD), which converts glutamate into gamma amino butyric acid, resulting in a loss of excitotoxic protection postnatally.[345]

Urena-Guerrero and co-workers found that MSG treatment neonatally in rats reduced GAD activity in the cerebral cortex at PND 21 and 60 due to decreased enzyme affinity.[346] Hippocampal and cerebellar areas demonstrated an upregulation of GAD in response to GABA neuron loss.

In an earlier study, Beas-Zarate and co-workers exposed rats to MSG at day 1,3,5 and 7 postnatally and assessed GABA release on day 14,21,30 and 60 utilizing [^{3}H]-GABA radiolabeling.[347] They found a major decline in [^{3}H]-GABA release under baseline conditions in the cerebral cortex at day 30 and 60. Stimulated GABA release was suppressed in the cerebral cortex on days 14 and 21 and significantly increased on day 60. In the hippocampus GABA secretion was suppressed on day 14, 21 and 60 and on all days in the striatum.

From these studies it is reasonable to assume that exposure to glutamate and aspartate food additives can significantly worsen the neurotoxic conditions seen in the child with autism spectrum disorders, including microglial activation, neurodegeneration, abnormal pathway development, inflammatory cytokine release, free radical and lipid peroxidation damage and mitochondrial dysfunction.

Other Environmental Toxins of Concern

There are other neurotoxic substances in the environment of autistic children that should be of concern, such as aluminum, cadmium, lead and fluoride. I will confine my remarks to aluminum and fluoride, since these are rarely considered.

Beside vaccines, aluminum is found in a number of baked goods (pancakes and biscuits), processed cheeses and teas. In one study food was found to supply 25-fold more aluminum systemically than public drinking water.[348] Recent studies have shown very high levels in a number of commonly used feeding and intravenous parenteral solution used in pediatrics.[349,350] Aluminum not only accumulates in the brain but produces inflammation, free radical generation and interferes with neuronal tubular function.[351,352]

Mundy and co-workers found that aluminum potentiated glutamate excitotoxicity and by increasing iron entry into neurons, increased ROS formation.[353]

Another neurotoxin of concern is fluoride, which has been shown to accumulate in the brain and trigger ROS and LPO accumulation as well as inhibition of critical antioxidant enzymes.[354,355] It has also been linked to an excitotoxic reaction within the brain.[356] Mullenix et al demonstrated significant behavior abnormalities in rats exposed to fluoride in drinking water, which were sex and dose dependent.[357] Males were more sensitive to prenatal exposure on days 17-19 and females where more sensitive to weaning and adult exposures. The doses were comparable to that seen in humans. They found the greatest fluoride accumulation to occur in the hippocampus, that was time and dose dependent.

Sources of fluoride include using fluoridated water used to reconstitute baby formula, fluoride drops and contaminated foods, especially processed meats and teas. Additive, and even synergistic toxicities of these food additives are known to occur.

Conclusion

There is compelling evidence that excessive immune stimulation during critical stages of brain development can cause disruption of neurodevelopment by affecting neuronal and glial cell migration, dendritic outgrowth, synaptic development and consolidation and by triggering neurodegeneration. Primarily involved are abnormalities in calcium homeostasis cause by stimulation of various receptors via inflammatory cytokines, glutamate receptors and androgen excess.

Likewise, there is an intimate interrelationship between excitotoxicity, inflammatory cytokines, free radical generation, lipid peroxidation and abnormalities in calcium homeostasis, which occur in an autocrine manner. Central in this process is dysfunction of mitochondria, which also increases the generation of free radicals, particularly peroxynitrite and dramatically increases sensitivity to extracellular glutamate to the extent that even physiological levels of glutamate can be excitotoxic.

As chronic inflammatory change takes place in the brain, secondary suppression of mitochondria occurs as a result of ischemia/hypoxia. This also increases sensitivity to excitotoxicity by reducing cellular energy production, which further aggravates disruptions of calcium homeostasis. In the developing brain a loss of calcium oscillation caused by excitotoxic and androgen-induced calcium excess, impairs progenitor cell migration and differentiation.

Increasing energy production, utilizing coenzyme Q10, L-carnitine, alpha-lipoic acid and other metabolic precursors and substrates, can significantly reduce glutamate excitotoxic damage.[358] Likewise, there are a number of natural products that inhibit glutamate receptors and reduce excitotoxicity.

Neurotoxic metals, such as mercury, aluminum and fluoride further aggravate the above processes as described. Mercury is a particularly potent inhibitor of the glutamate transporters, resulting in rising levels of extracellular glutamate and by inhibiting antioxidant enzymes and glutathione, aggravates free radical generation and the accumulation of lipid peroxidation products.

The glutamate receptors are known to exist on other neuron systems, such as serotonergic[359], cholinergic[360] and dopaminergic neurons[361,362], thus exercising widespread control of complex neural networks in the prefronto-limbic brain, areas significantly affected in autism spectrum disorders.

Finally, recent studies have identified a number of common genetic abnormalities in autistic spectrum disorders, all of which either regulate glutamatergic receptors or GABA receptors.[363,364] The GABA receptors normally play a role in controlling glutamate receptor overactivity.

Of particular interest is the study by Jamain and co-workers who studied a large number of autistic subjects and found a strong linkage between GluR6 gene and autism.[365] Abnormalities in the GluR6 gene may be related to impaired communication and learning in autistics. GluR6 is abundantly expressed in the hippocampus, basal ganglion and cerebellum, areas affected in autism spectrum disorders. It has also been suggested that abnormalities in GluR6 may account for the increased incidence of seizures in autistic children.[366]

Another gene mutation of interest involves reelin, a large polypeptide that plays a major role in neurodevelopment by directing laminar architechtonic construction and at later stages, plasticity. Several studies have found abnormalities in the genes controlling reeler secretion in the developing brain[367,368], even though others found no association.[369] It is of interest that reelin appears in the neocortex in humans around the 5th week of gestation, which coincides with the time window of autism induced by agents.

Deficiencies in reelin are associated with such abnormalities as inverted cortical lamination, aberrant position of neurons and decreased connectivity, similar in many ways to the pathological findings in autism. Abnormalities in reelin during critical periods of brain development have been associated with other neurodevelopmental and psychiatric disorders, including schizophrenia and bipolar disorders.[370]

Even without genetic polymorphisms as a cause of reelin disruption during development, other factors are known to affect reelin levels, such as NT4, BDNF and thyroid hormone. Studies have demonstrated a 43 to 44% reduction in reelin levels in cerebellar homogenates of autistic brains as compared to controls[371] as well as reduced blood levels of reelin.[372]

As we have seen, prenatal viral infections are associated with autistic-like syndromes in offspring. Fatemi and co-workers have shown that prenatally infected mice demonstrate defective cortical laminar development and a reduction in reelin immunoreactivity in the affected areas.[373] This strongly suggest that inflammatory cytokines are the culprit in reducing reelin levels because, as demonstrated, these viruses are not transferred to the fetus, whereas the cytokines are. In addition, reelin has been shown to increase the sensitivity of the NMDA and AMPA receptors in the brain.[374]

It appears that the autistic process begins with microglial activation, which becomes chronic. Priming of the microglia can occur as a result of immune system dysfunction coupled with recurrent immune stimulation, either secondary to vaccination or recurrent systemic infections or both. Once primed, the microglia, upon further stimulation, produce an exaggerated response in terms of inflammatory cytokine release and release of the excitotoxins, glutamate and quinolinic acid, from microglia and astrocytes. Further stimulation of systemic immunity, again either by recurrent systemic infections or closely

spaced or an excessive number of vaccinations, reactivate the primed microglia, leading to the autotoxic bystander damage.[227]

This microglial hyperimmunity is also driven by food peptide allergies and Candida infections as well as other ingested toxins such as food-excitotoxin additives, fluoride and aluminum. Elimination of all of inflammatory cytokines, androgenic excess, neurotoxic metals and excitotoxic additives may be necessary, early in the course of the disorder, in order to salvage the child's neurological function.

While inflammatory cytokines can alter behavior acutely or chronically, excitotoxicity still plays a major role in the effects of cytokines on higher cerebral function. Despite the fact that microglia secrete large amounts of two known excitotoxins, glutamate and quniolinic acid, few studies on autism have mentioned this as a major mechanism for the changes seen in the autistic brain. Even though the concentration of the secreted glutamate and quinolinic acid alone are sufficient to elicit excitotoxicity, the fact that in the autistic brain we see mitochondrial dysfunction, high levels of oxidative stress, low magnesium and high levels of TNF-α and IL-1ß, conditions that magnify glutamate excitotoxicity, should call attention to this being a major mechanism in damage to the autistic brain.

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