"Oxidation and Epigenetics in Human Brain: Implications for Vaccination"

The ability to resist oxidation is a central feature of all living organisms. The tripeptide glutathione, whose synthesis is limited by availability of cysteine, is the primary intracellular antioxidant and the ratio of reduced glutathione (GSH) to its oxidized dimeric counterpart (GSSG) is a central determinant of the cellular redox state. To maintain the GSH/GSSG ratio, electrons arising from glucose-6-phosphate and isocitrate are transferred to $NADP^+$ (*1*) and NADPH provides them directly to GSSG via glutathione reductase, or indirectly via the selenoprotein thioredoxin reductase (*2*). Mitochondrial oxidative phosphorylation is the primary source of reactive oxygen species (ROS), and the level of GSH must be maintained at a sufficient level to offset ROS production, otherwise cells experience oxidative stress, which adversely affects a myriad of metabolic processes and degrades cell function.

Evolution has provided cells with an array of adaptive responses to oxidative stress, which can restore redox equilibrium, but not without metabolic consequences. An important example is the folate and vitamin B12 (cobalamin)-dependent enzyme methionine synthase (MS), which is exquisitely sensitive to cellular redox state (*3*), and when its cobalamin cofactor is oxidized, its substrate, homocysteine is diverted to increase cysteine and GSH levels (*4*), helping to restore equilibrium (Fig. 1). However, MS is the master controller of more than 150 methylation reactions, each of which is inhibited in parallel with lower MS activity. Key among these reactions is methylation of DNA, which is the key event underlying epigenetic regulation of gene expression, and epigenetic regulation is fundamental to development (*5*). This reciprocal relationship between redox and methylation implies that agents capable of promoting oxidative stress will also produce developmental toxicity (*6*).

MS activity is also essential for dopamine-stimulated phospholipid methylation, an exclusive activity of the D4 dopamine receptor (*7*) (Fig. 1, lower left). This activity appears to be involved in gamma frequency synchronization of neural networks during attention (*8*), and specific genetic variants of the D4 receptor are associated with increased risk of attention-deficit hyperactivity disorder (ADHD) (*9*). Interestingly, ADHD shows a 4-fold higher prevalence in males than females, similar to the prevalence for autism, and the D4 receptor is the only known substrate for MS other than homocysteine (*7*). As such, dopamine-stimulated phospholipid methylation is particularly vulnerable to impaired MS activity secondary to oxidative stress.

The brain utilizes oxygen at a 10-fold higher rate than other tissues, increasing its dependence upon antioxidant supply and placing its development at greater risk. Despite this increased demand for antioxidant, cerebrospinal fluid levels of cysteine are 100-fold lower than levels in plasma (*10*), and neurons are dependent upon cysteine released from neighboring astrocytes to meet their redox needs. The transsulfuration pathway which converts homocysteine to cysteine, is only partially active in brain, and is less active in human brain than in other species (*11*), increasing the reliance of neurons on cysteine uptake, as illustrated in Figure 1. Recent studies in our laboratory reveal that neurotrophic growth factors such as insulin-like growth factor (IGF-1) and brain-derived neurotrophic factor (BDNF) promote neuronal uptake of cysteine by increasing activity of excitatory amino acid transporter-3 (EAAT3), which is the primary source of neuronal cysteine. Growth factor stimulation of EAAT3 is mediated by their activation of the PI3 kinase signaling pathway, and results in an increase in GSH/GSSG, as well as an increase in MS activity, reflected as an increase in the ratio of the methyl donor S-adenosylmethionine (SAM) to the methylation inhibitor S-adenosylhomocysteine (SAH). This represents

a redox-based signaling pathway which allows growth factors to exert an epigenetic influence via the intermediate activation of MS. Disruption of this pathway will not only contribute to impaired neurodevelopment at younger ages, but may also promote neurodegeneration in old age, since neuronal survival is dependent upon ongoing neurotrophic growth factor stimulation.

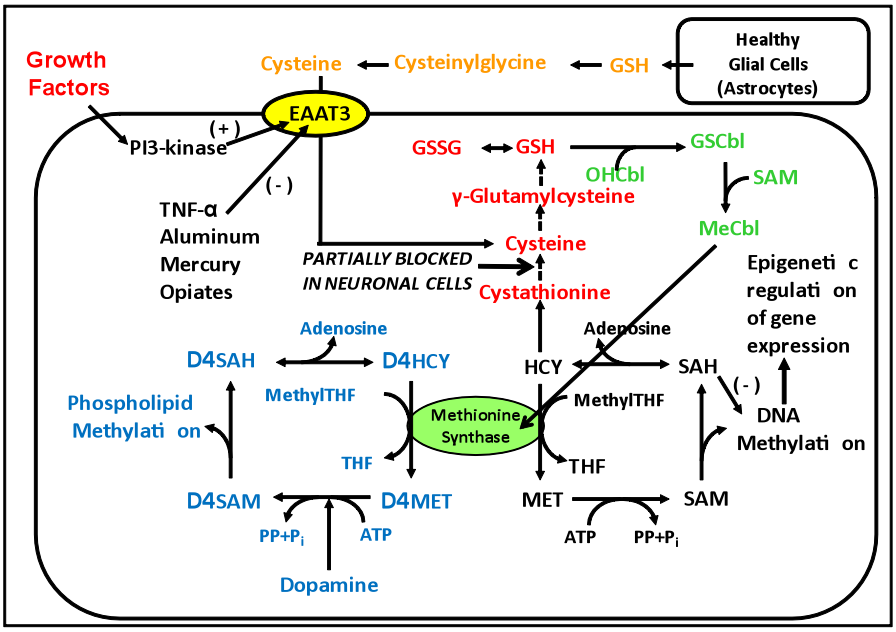

Figure 1: Redox and methylation metabolic pathways in neuronal cells. The primary source of neuronal cysteine is EAAT3-mediated uptake, which is increased by growth factors but decreased by aluminum and mercury, and by opiates, including casein and gluten-derived opiate peptides.

Using qRT-PCR with mRNA probes directed toward the cobalamin-binding domain, our laboratory recently evaluated the status of MS mRNA in postmortem human cortex of control subjects ranging in age from late gestation (28 weeks) to 84 yrs. We found a remarkable pattern of progressive decrease in the level of MS mRNA, which amounted to 400-fold across the lifespan (*12*). This previously undescribed decrease indicates that MS transcription is gradually reduced with age, and the consequential decrease in MS activity will result in a gradual increase in transsulfuration of homocysteine and increased GSH synthesis. This pattern of increased GSH synthesis is a very useful adaptive response to maintain redox equilibrium in the face of increasing ROS formation with advancing age. The latter has been attributed to accumulating mitochondrial DNA damage with age. A further comparison using PCR probes directed against the Cap domain of MS showed an overall pattern of decrease similar to that of the cobalamin-binding domain, although the decrease in older age subjects was significantly greater such that little or no Cap domain mRNA could be detected in subjects > 70 yrs of age (*12*). The age-dependent loss of Cap domain reflects alternative splicing of MS pre-mRNA resulting in the exclusion of certain exons, a process that is known to be more prevalent in the brain. The Cap domain normally limits oxidative inactivation of MS and its deletion will therefore increase sensitivity, leading to increased GSH formation. Cultured cell studies of the alternatively spliced form of MS reveal that the “Cap-less” enzyme has an absolute requirement for methycobalamin (methylB12), whose synthesis is GSH-dependent, as illustrated in Fig. 1 (upper right). Thus human brain exhibits

several adaptive mechanisms involving MS which combine to maintain GSH levels with increasing age. However, these unique features make the human brain more highly sensitive to factors that interfere with GSH formation, including inflammatory cytokines produced during the immune response and metals that are commonly incorporated into vaccines.

Tumor necrosis factor-α (TNF-α) is a well-characterized pro-inflammatory cytokine whose levels increase as part of the immune response to vaccination (*13*). We found that TNF-α causes a prompt and significant decrease in EAAT3-mediated cysteine uptake in cultured human neuroblastoma cells, in conjunction with a decrease in MS activity and an increase in cystathionine and GSH levels. These changes indicate that TNF-α shifts the source of cysteine away from the growth factor-sensitive EAAT3 transport while increasing transsulfuration of homocysteine.

Aluminum, in the form of alum (aluminum hydroxide) is included in most vaccines as an adjuvant to augment the immune response (*14*). Recent studies have attributed its adjuvant response to increased levels of ROS (*15*), which may result from inhibition of mitochondrial NADPH formation by isocitrate dehydrogenase (*16*). We found that aluminum potently decreases EAAT3 activity, lowers GSH levels and inhibits MS activity in neuroblastoma cells, resulting in decreased methylation activity, including decreased phospholipid methylation (*17*).

Mercury, in the form of thimerosal (ethylmercury thiosalicylate), has been used as a vaccine preservative and remains in certain vaccines (e.g. most seasonal flu vaccines), although it has been removed from infant vaccines, except for trace amounts. Mercury binds with exceptional strength to thiols and especially to selenoproteins such as thioredoxin reductase, to form essentially irreversible inactive complexes (*18*) . Thimerosal also potently inhibits EAAT3-mediated cysteine uptake, lowers GSH levels, inhibits MS activity and impairs methylation, being somewhat more potent than aluminum.

Many autistic children are benefited by a gluten-free/casein-free diet (*19*), and gluten intolerance is becoming an increasingly common complaint (*20*). In addition, several recent studies have demonstrated that casein consumption contributes to autoimmunity (*21*, *22*). We therefore investigated a possible influence of gluten and casein-derived peptides on redox and methylation status and found that very similar proline-rich peptides from each food source caused inhibition of EAAT3-mediated cysteine uptake in both human GI epithelial cells and neuroblastoma cells. This effect was mediated by their stimulation of mu-type opiate receptors, which could be blocked by naloxone and naltrexone. Interestingly, naloxone has been shown to be as effective as aluminum as a vaccine adjuvant (*23*).

Taken together he above findings indicate that vaccines and the immune reaction they provoke will produce a powerful oxidative stress response that not only involves immune cells, but may also impact neuronal function, especially in the developing brain. The ability of TNF-α and peripherally administered aluminum and mercury to enter the brain is well-documented (*24*, *25*), and both metals can be retained in the brain for many years. Based upon these actions, it is reasonable to expect that vaccines may be an important contributing factor for the dramatic increases in autoimmune and neurodevelopmental disorders occurring over the past several decades. In addition to shedding light on the mechanism(s) by which vaccination may cause disease in susceptible individuals, our studies highlight the opportunity to identify vaccine components (i.e. adjuvants and preservatives) which are less likely to provoke these responses (i.e. prolonged oxidative stress) and less likely to affect neuronal development. Incorporating such improved agents into vaccines will not only improve their safety, but will also increase public confidence in vaccination programs.

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