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Review

Non-protein amino acids and neurodegeneration: The enemy within

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ABSTRACT

Animals, in common with plants and microorganisms, synthesise proteins from a pool of 20 protein amino acids (plus selenocysteine and pyrolysine) (Hendrickson et al., 2004). This represents a small proportion (~2%) of the total number of amino acids known to exist in nature (Bell, 2003). Many 'non-protein' amino acids are synthesised by plants, and in some cases constitute part of their chemical armoury against pathogens, predators or other species competing for the same resources (Fowden et al., 1967). Microorganisms can also use selectively toxic amino acids to gain advantage over competing organisms (Nunn et al., 2010). Since non-protein amino acids (and imino acids) are present in legumes, fruits, seeds and nuts, they are ubiquitous in the diets of human populations around the world. Toxicity to humans is unlikely to have been the selective force for their evolution, but they have the clear potential to adversely affect human health. In this review we explore the links between exposure to non-protein amino acids and neurodegenerative disorders in humans. Environmental factors play a major role in these complex disorders which are predominantly sporadic (Coppede et al., 2006). The discovery of new genes associated with neurodegenerative diseases, many of which code for aggregation-prone proteins, continues at a spectacular pace but little progress is being made in identifying the environmental factors that impact on these disorders. We make the case that insidious entry of non-protein amino acids into the human food chain and their incorporation into protein might be contributing significantly to neurodegenerative damage.

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Toxicity of non-protein amino acids

Some non-protein amino acids are able to compete in metabolic pathways involving protein amino acids: in other words, they function as antimetabolites (Rubenstein, 2000). They can also be mistakenly utilised in protein synthesis ('proteomimetics') (Fowden et al., 1967; Rodgers and Shiozawa, 2008; Rubenstein, 2000). Incorporation of non-protein amino acids (amino acid analogues) into proteins was systematically studied in the 1960s but this phenomenon and its possible implications have been largely overlooked (reviewed in (Hendrickson et al., 2004)). An exception is the arginine-mimetic canavanine, which occurs in Papilionoideae such as the jack bean, and kills the larvae of predators by replacing arginine in the peptide chain of newly-synthesised proteins (Thomas and Rosenthal, 1987). Canavanine, if supplied in a high enough concentration, is lethal to rats (Thomas and Rosenthal, 1987) and is currently being investigated as a human anti-cancer agent (Vynnytska et al., 2010).

In other examples, fescue grasses out-compete other plants by releasing a phytotoxic root exudate of which *meta*-tyrosine, a proteomimetic amino acid, is the primary component (Bertin et al., 2007). *Meta*-tyrosine interferes with root development in competing

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plants by being charged to $tRNA^{phe}$ and replaces phenylalanine in the protein sequence (Bertin et al., 2007). *Meta*-tyrosine is also a product of phenylalanine oxidation and is utilised by mammalian cells *in vitro*, resulting in the synthesis of aberrant proteins which are toxic or lethal (Rodgers et al., 2002). Similarly, the proline mimetic azetidine-2-carboxylic acid, which is synthesised by *Convallaria majalis*, is lethal to other plant species that do not synthesise this imino acid and competes with proline for insertion into proteins (Peterson and Fowden, 1965).

Use of non-protein amino acids in translation of mRNA to protein

Proteomimetic amino acids, being close structural analogues of the 20 protein amino acids, enter mammalian cells and cross the blood–brain barrier on amino acid transporters and once inside the cells compete with protein amino acids for charging onto tRNA (Hendrickson et al., 2004). A high level of fidelity in protein synthesis is maintained because of a very low error rate on the part of tRNA synthetases when selecting the correct protein amino acid for esterification to the cognate tRNA and from the abortive termination of protein synthesis following detectable errors in this process (Zaher and Green, 2009). tRNA synthetases have evolved to discriminate efficiently between the 20 protein amino acids, and in some cases require an additional proof-reading step (Hendrickson et al., 2004) but are less efficient at discriminating against non-protein amino acids with a similar, size, shape and charge to a protein amino acid (Fowden et al., 1967; Hendrickson et al., 2004). For example, L-3,4 dihydroxyphenylalanine (L-DOPA), a very close structural analogue of L-tyrosine, can be charged to $tRNA^{tyr}$ in mammalian cells resulting in the synthesis of full-length proteins containing biosynthetically incorporated L-DOPA (Rodgers et al., 2002).

The most effective defence against proteomimetic amino acids is to evolve a more selective tRNA synthetase. The bruchid beetle, a canavanine-resistant predator, has adopted this strategy, possessing an advanced arginyl tRNA synthetase which has a low affinity for canavanine relative to arginine (Malinow et al., 1982; Rosenthal et al., 1976). Similarly, the jack bean has been shown to possess an advanced arginyl tRNA synthetase that prevents auto-toxicity from canavanine insertion into its own proteins (Igloi and Schiefermayr, 2009).

Specific effects of proteomimetic amino acids on human health

Proteomimetic amino acids impact on human health only when high enough quantities are ingested to compete effectively with the 'parent' protein amino acid for insertion into polypeptide chains. In humans, exposure to canavanine from ingestion of alfalfa seeds or tablets can cause systemic lupus erythematosus (SLE)-like symptoms, an effect also seen experimentally in cynomolgus macaques (Malinow et al., 1982). Similarly, ingestion of 1,1′-ethylidene-bis[L-tryptophan] (EBT), a contaminant in a synthetic tryptophan preparation, resulted in over 1500 cases of Eosinophilia–Myalgia Syndrome (EMS) and 38 deaths (Rubenstein, 2000). Administration of EBT to rats resulted in the development of EMS and confirmed that the amino acid was incorporated into proteins (Silver et al., 1994). The L-tyrosine mimetic L-DOPA, which is present in seeds from the highly insect-resistant Central American plants of the genus *Macuna* (at ~6–9%), is incorporated into proteins by mammalian cells in place of L-tyrosine (Rodgers et al., 2002, 2004) and generates protease-resistant, aggregate-prone proteins in human cells *in vitro* (Dunlop et al., 2008; Rodgers et al., 2004).

Incorporation of a proteomimetic amino acid into proteins is a random process and, although no specific proteins are targeted, aggregation-prone or 'intrinsically disordered proteins' (IDP) such as α-synuclein and tau protein could be more sensitive to an amino acid substitution (Uversky et al., 2008). The extent to which an amino acid substitution destabilises a protein depends, amongst other factors, on which amino acid is substituted and its location in the folded protein; internal or solvent-exposed (Ozawa et al., 2005). Post-mitotic cells such as neurons and retinal pigment epithelial (RPE) cells are less well-equipped to handle terminally aggregated proteins than rapidly dividing cells, since they are unable to reduce the burden of protein aggregates by distributing them amongst daughter cells. In these ways global errors in protein synthesis can cause tissue-specific dysfunction (Drummond and Wilke, 2009), as was evident in a study in which neurodegeneration was the primary pathology in a mouse with a minor translational proof-reading defect (Lee et al., 2006). Proteins which have been implicated in neurological disorders are generally aggregation-prone or contain domains with a high aggregation propensity (King et al., 2012): mutations in genes encoding these proteins, however, are not always 'causative' for the disorder to which they are linked, so are considered to be 'susceptibility genes' that by themselves do not give rise to a phenotype (Rocchi et al., 2003). Incorporation of proteomimetic amino acids into proteins could therefore be an environmental trigger which accelerates the rate of protein aggregation in individuals carrying susceptibility genes.

Proteomimetic amino acids and neurological disease

Sufferers from Parkinson's disease are exposed to the L-tyrosine mimetic L-DOPA (levodopa) over many years as a therapeutic agent for their medical condition. L-DOPA is efficiently incorporated into proteins by mammalian cells *in vitro* (Rodgers et al., 2002) and proteins containing incorporated DOPA are present in blood and brain extracts from L-DOPA-treated individuals (Chan et al., 2012; Rodgers et al., 2006). Despite being in use for over 40 years there is still much debate about whether L-DOPA might accelerate the progression of Parkinson's disease (Zesiewicz, 2011) this has been hard to resolve since almost all Parkinson's patients are eventually treated with L-DOPA. It is likely that oxidative stress from L-DOPA is an *in vitro* artefact (Clement et al., 2002), however, proteins containing incorporated L-DOPA are cytotoxic *in vitro* (Chan et al., 2012) and would be capable of producing a slowly progressive chronic toxicity *in vivo*. When present in proteins or other polymers, L-DOPA is one of nature's most effective adhesives and cross-linking agents (Messersmith, 2010; Miserez et al., 2008) and is capable of generating protein aggregates in cells preventing their removal by proteolysis (Dunlop et al., 2008).

The non-protein amino acid β-methylamino-L-alanine (BMAA) has been linked to neurological diseases. BMAA has been implicated in a complex neurological disorder on the South Pacific island of Guam in which individuals (from different cultural and genetic backgrounds) slowly developed features of amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and dementia (known collectively as ALS-PDC) (Bradley and Mash, 2009). This disorder occurred at a 100-fold greater incidence than that of ALS in the USA and other developed countries (Kurland, 1988). Steele and Guzman reported a higher incidence of ALS–PDC in villages in the south of Guam where cycad flour was eaten more frequently and made the observation that Chamorro people on the nearby island of Saipan, who did not consume cycad flour, had no increased incidence of ALS–PDC (Steele and Guzman, 1987). This supported Whiting's hypothesis that seeds of *Cycas circinalis* contained a neurotoxin (Whiting, 1963). Although cycad flour contained acute toxins that caused diarrhoea, vomiting and in some cases death (Whiting, 1963), these were generally removed prior to use by an extensive (but variable) washing procedure, leaving lipid soluble substances as well as insoluble or protein-bound material (Whiting, 1963). Washed cycad flour has a complex toxicity profile in mice that includes glutamate release, gait disturbances, loss of muscle strength and balance, as well as apoptotic cell death in the spinal cord and brain (Shaw and Wilson, 2003). Acute toxins such as sterol glucosides remain in the flour after washing and appear to contribute significantly to the excitotoxic effects of the washed cycad flour (Khabazian et al., 2002). The amino acid BMAA was identified in cycad seeds by Bell et al. (Vega et al., 1968) and in a landmark study, Spencer demonstrated that administration of BMAA to cynomolgus monkeys resulted in the development of a degenerative motor-system disease (Spencer et al.,