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## Glutathione Metabolism and Parkinson's Disease

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### Abstract

It has been established that oxidative stress, defined as the condition when the sum of free radicals in a cell exceeds the antioxidant capacity of the cell, contributes to the pathogenesis of Parkinson's disease. Glutathione is a ubiquitous thiol tripeptide that acts alone, or in concert with enzymes within cells to reduce superoxide radicals, hydroxyl radicals and peroxynitrites. In this review, we examine the synthesis, metabolism and functional interactions of glutathione, and discuss how this relates to protection of dopaminergic neurons from oxidative damage and its therapeutic potential in Parkinson's disease.

### Keywords

Glutathione; Glutathione S-transferase; Parkinson's disease; Oxidative Stress; Substantia nigra

### Introduction

Neurons are among the most metabolically active cells in the body, requiring the correct balance of oxygen and glucose to maintain healthy function. However, when the metabolic balance is overwhelmed and the sum of free radicals in a cell is greater than the capacity of the cell to detoxify these substances, oxidative stress is generated. Increased oxidative stress has been shown to contribute to the etiology or progression of a number of neurodegenerative diseases since the brain uses a disproportionate amount of oxygen per volume of tissue compared to other organs [1]. When free radicals of oxygen are present within the environment of the cell, they may damage lipid membranes, interfere with DNA integrity, and interrupt cellular respiration through alterations in mitochondrial complex I [2–4]. The reduction or detoxification of free radicals is handled by a number of homeostatic mechanisms, in normal physiological conditions.

Parkinson's Disease (PD) is one of the neurological disorders affected by changes in oxidative balance. PD is a progressive neurodegenerative disease with noticeable outward symptoms generally appearing in the sixth decade of life. The most common phenotypes of

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this disorder include progressive deterioration of autonomic and motor functions and in some cases, cognitive decline. Although the underlying etiology of Parkinson's disease is not completely understood [5, 6], the most common neuroanatomical pathology is the accumulation of misfolded alpha-synuclein into intracellular aggregates called Lewy Bodies, present throughout the enteric [7, 8], peripheral [9] and central nervous systems [10, 11]. Progression of the disease results in the significant loss of the dopaminergic neurons situated in the midbrain substantia nigra pars compacta.

## Sources of Reactive Oxygen Species in the Substantia Nigra

The loss of dopaminergic neurons located in the substantia nigra pars compacta (A9) is the lesion most characteristic of Parkinson's disease, although other regions of the central, peripheral and enteric nervous systems also show considerable cell loss [12–15]. Within the CNS, it is not entirely clear why the substantia nigra is so significantly affected, although this region does have a number of characteristics that make it particularly vulnerable to oxidative stress. These factors include (but are not limited to) the presence of endogenous dopamine, iron, and neuromelanin [16–18]. Additionally, the intrinsic antioxidant defenses in this structure are more vulnerable than in other brain regions due to lower levels of glutathione (GSH) [19, 20] and glutamylcysteine ligase activity [21], and higher microglial:astrocyte ratios [22, 23].

Dopamine (DA), which is the most abundant neurotransmitter in the basal ganglia [24], is synthesized in the large diameter neurons of the substantia nigra and is released from the terminals that reside within the caudate and putamen nuclei (in rodents this is called the striatum) [25]. Functionally, dopamine modulates excitatory and inhibitory synaptic transmission ensuring smooth directed movement [26]. When released from presynaptic terminals, DA is actively taken up from the synaptic cleft through a number of monoamine transporters (i.e dopamine active transporter (DAT)), where it is packaged into intracellular vesicles by vesicular monoamine transporters (VMAT) [27]. In the SNpc dopaminergic neurons, the predominant VMAT is VMAT2 [28, 29]. When DA is produced in excess of capacity and cannot be transported into the cell through the DAT or packaged internally by VMAT, it remains in free form where it can be readily oxidized to DA quinone, or form superoxides and hydrogen peroxide [30–32]. These superoxides may damage cell and organelle membranes, leading to cellular dysfunction.

Inside the cell, DA quinones react with the sulfhydryl groups of the free amino acid cysteine, cysteine found in glutathione, and other cysteine residues to covalently modify proteins [31, 32] that cause cellular toxicity and in some cases, cell death [30, 31, 33, 34]. DA quinones have also been shown to react with neuromelanin to form eumelanin [35], which is present in DA neurons of the SN. DA may also autooxidize to form hydroxyl radicals (OH<sup>•</sup>) [30, 32, 36] or after oxidation to hydrogen peroxide, may react with iron, copper, or oxygen (O<sub>2</sub>) to form hydroxyl radicals [37].

Iron metabolism is necessary for the function of some enzymes, including tyrosine hydroxylase (the rate limiting enzyme in DA biosynthesis) and for overall neuronal health [38–41]. Iron is transported into cells from the bloodstream while bound to transferrin, and stored intracellularly by binding to the protein ferritin [37]. Ferritin in the cytosol is comprised of heavy (H) and light (L) chain subunits. The H-subunit has ferroxidase activity, converting Fe<sup>2+</sup> to Fe<sup>3+</sup>, while the L-subunit stabilizes the complex of subunits to remain in iron storage form. The ratios of H- versus L-type subunits of ferritin vary among tissues and in different cell types within the brain. These differences can affect the interactions of iron with other cellular components and make some cell types more vulnerable to oxidative stress [37, 42].

Within the CNS, the SN is the structure containing the highest level of iron [43, 44]. In a reduced state, iron ( $\text{Fe}^{2+}$ ) readily reacts with hydrogen peroxide to form hydroxyl radicals via the Fenton reaction [37, 45]. The ratio of reduced iron ( $\text{Fe}^{2+}$ ) to oxidized iron ( $\text{Fe}^{3+}$ ) is approximately 1:1 in the normal SN [46, 47]. However, in PD patients the ratio of reduced to oxidized iron in the SN has been reported to increase [48], in one report to 1:3 [49]; a dysregulation not found in other tissues or regions of the brain [49, 50]. Since numerous studies have shown that the elevated levels of reduced iron in the SN can lead to cellular toxicity [51–54], it has been suggested that iron chelation may provide some level of neuroprotection in Parkinson's disease [55–58].

The SN contains another protein that may also contribute to oxidative stress. Neuromelanin, a brown, black insoluble substance that is formed from oxidative metabolites of dopamine and norepinephrine [59, 60], has been shown to interact with lipids, pesticides, other toxic compounds including paraquat [61, 62], and many heavy metal ions including iron [63–65]. Of the transition metals, neuromelanin binds most tightly with iron [62, 65]. Although these interactions may initially be protective [66], when this system is overwhelmed (i.e. iron is present in excess), neuromelanin may begin to catalyze the production of free radicals [67].

## Glutathione: An Important Antioxidant in the Brain

Glutathione (GSH), a ubiquitous thiol tripeptide, provides protection from oxidative stress-induced damage through the reduction of reactive oxygen species (ROS). GSH acts alone or in concert with other enzymes to reduce superoxide radicals, hydroxyl radicals and peroxynitrites [3]. Additionally, GSH detoxifies xenobiotics, is a storage and transfer form for cysteine, and maintains cellular redox potential by keeping sulfhydryl proteins in a reduced state [68]. The antioxidant characteristics of GSH have been demonstrated in a number of models of oxidative stress including depletion of GSH with L-buthionine-(S,R)-sulphoximine (BSO) [69–73], ethacrynic acid [74], or reduction of GSH synthesis using antisense directed against gamma-glutamylcysteine synthetase ( $\gamma$ GCS), hereafter referred to as glutamylcysteine ligase (GCL) (see section on GSH synthesis below) [75–78] or glutaredoxin 2 [79]. In these studies, diminished levels of GSH increase oxidative stress in whole cells as well as in mitochondrial fractions, and increase lipid peroxidation, intracellular calcium, and gamma-glutamyl transpeptidase ( $\gamma$ GT) activity.

Several studies discussed below illustrate these points by utilizing dopaminergic systems. Depletion of GSH by BSO, an irreversible inhibitor of *GCL*, that does not by itself induce nigrostriatal damage in vivo [80], potentiates the amount of MPTP-induced tyrosine hydroxylase-positive ( $\text{TH}^+$ ) neuron death in the SNpc (48.6% cell death compared to 30.1% cell death) [69, 80]. Additionally, in conditions of increased oxidative stress such as when mesencephalic cells are placed in culture or during normal ageing in vivo, decreasing GSH levels causes neuron loss [76].

The reduction of GSH activity by ethacrynic acid (EA), an effective loop diuretic used in clinical practice [81], has also been shown to increase cell sensitivity to free radicals. Astrocytes exposed to EA and 3-morpholinopyridone (SIN1, a compound that generates peroxynitrites) show significant increases in lactate dehydrogenase (an indirect marker of cell death), decreased ATP levels and decreased mitochondrial membrane potential. The critical role of GSH in this system is shown by experiments where excess GSH monoester is included with EA and SIN1, and LDH activity is inhibited [74]. Treatment with a thiol ester compound (gamma-glutamylcysteinyl ethyl ester) or exogenous GSH while examining cellular models of diminished GSH levels in concert with Complex I inhibitors  $\text{MPP}^+$ , MPTP, or DHBT-1, restores mitochondrial Complex I activity, inhibits cell loss and protects against cell and striatal DA loss [73, 75, 82].

Further support for the critical role of GSH as a free radical reducing agent is demonstrated in several studies that utilize siRNA knockdown of GSH modulating enzymes. Lee et al [79] used siRNA directed against glutaredoxin 2 (Grx2), a protein that is critical for controlling redox signaling in the mitochondria by modulating the interactions between the glutathione pools and protein thiols [83] to reduce Complex I activity [84]. The reduction in Grx2 results in a loss of Complex I (30%) and m-aconitase (60%) activity, decreased ferritin levels and a subsequent increase in mitochondrial iron [79]; each leading to increased oxidative stress. Similarly, a 50% reduction of GSH by antisense knockdown of GCL [77] increases cellular oxidative stress in PC12 cells. Using this paradigm, Jha et al [78] noted a reduction in mitochondrial performance using three different measures; a 70% reduction of ATP levels, a 65% reduction in mitochondrial pyruvate-dependent 5,5'-dithiobis (2-nitrobenzoic acid) (MTT), and a 60% reduction in oxygen consumption. It was also demonstrated that this effect was specific to complex I, and not complex II, III, or IV activity. Addition of dithiothreitol (a thiol reducing agent) restored complex I activity to control levels in GCL-diminished cells, suggesting that it is the oxidation of sulfhydryl groups that results in the inhibition of complex I. In PC12 cells, GSH depletion decreases TH activity and DA uptake [77]. Additionally, mitochondrial complex I activity, which is reduced in Parkinson's disease and is critical to DA neuron survival [85, 86] is compromised when GSH levels are reduced in combination with exposure to NO and generation of peroxynitrite radicals [74, 75].

In the SN, the reduced form of GSH is an important mediator of oxidative stress. Studies of postmortem CNS of PD patients reveal lower levels of GSH in the SN (40%), but not in other regions of the brain, compared to age-matched controls. In contrast, GSH levels in the SN from patients with other neurodegenerative diseases that involve the basal ganglia, such as Multiple System Atrophy and Supranuclear Palsy, are unchanged [87].

## Glutathione Synthesis in the Brain

In non-nervous system mammalian cells, GSH is abundant and can be found at concentrations of 0.5–10 mM [68]. In the brain, however, GSH levels are often found at lower (1–3 mM) concentrations [88]; a protective mechanism itself, since the GSH precursor molecules may be toxic at high concentrations [89–92]. In general, the GSH tripeptide is synthesized as the product of two successive reactions (Figure 1). This synthesis occurs in neurons and glial cells, although astrocytes synthesize GSH more effectively than neurons based on their ability to utilize a wider variety of precursor substrates [93]. Neurons synthesize GSH using glutamine, glutamate, cysteine and glycine or conjugates of these amino acids provided by the breakdown of GSH by  $\gamma$ -glutamyl transpeptidase ( $\gamma$ GT). Astrocytes, however, have the ability to utilize a far greater number of substrates in the synthesis of GSH including the amino acids: glutamate, cysteine, glycine, glutamine, aspartate, asparagine, ornithine, proline and cysteine, conjugates of these amino acids as well as glutathione disulfide (GSSG), 2-oxothiadiazine-4-carboxylate (OTC), N-acetylcysteine (NAC) and GSH itself [3, 94–96], although each of these components are ultimately converted to glutamate, cysteine or glycine prior to GSH synthesis [97].

The first step in GSH synthesis is the formation of  $\gamma$ -glutamylcysteine ( $\gamma$ GC) from the substrates L-glutamate and L-cysteine by glutamylcysteine ligase (GCL) (also referred to as  $\gamma$ -GlutamylCysteine synthetase ( $\gamma$ GCS)) [98]. GCL is a heterodimer containing both disulfide and noncovalent bonds between its heavy and light chain subunits [99] is the enzyme in the rate-limiting step of GSH synthesis. Intracellular GSH levels are regulated by negative feedback of G by GSH [100] as well as the availability of cysteine [68]. An intermediate product of this synthesis,  $\gamma$ -glutamylphosphate, is produced by the reaction of L-glutamate and ATP, which reacts with L-cysteine to form L- $\gamma$ -glutamylcysteine. In the

second step of GSH synthesis, L- $\gamma$ -glutamylcysteine and ATP combine to form L- $\gamma$ -glutamyl-L-cysteinylphosphate that is combined with glycine in a reaction catalyzed by GSH synthase to form GSH [101, 102].

GSH is also metabolized to its component peptides that are recycled to regenerate GSH. In this reaction, the ectoenzyme  $\gamma$ -glutamyl transpeptidase ( $\gamma$ GT) catalyzes the transfer of the  $\gamma$ -glutamyl moiety from GSH or a GSH conjugate to an acceptor molecule (CysGly or CysGly conjugate) [95]. As mentioned above, glutathione disulfide (GSSG), 2-oxothiadolazine-4-carboxylate (OTC), and N-acetylcysteine (NAC) can also serve as precursors for the synthesis of GSH [95]. Alternatively, glutathione reductase (GR) uses NADPH as an electron donor to reduce GSSG and regenerate GSH [99].

## Reduction of Free Radicals by Glutathione Conjugating Enzymes

### Glutathione Peroxidase

Glutathione peroxidases (GPXs) are a group of 8 (GPX1-8) enzymes that are important for reducing hydrogen peroxide to water. GPX's are selenocysteine enzymes that use GSH as a reducing agent and require selenium [103, 104] for their antioxidant function. In the brain, the selenoproteins GPX-1-3 exist as tetrameric proteins, composed of four identical subunits with each monomer having a molecular weight of 22–23 kDa, [105], while GPX4 has activity as a monomer [106]. GPX1 and 4 are found in the mitochondria, nucleus and the cytosol [107], and GPX1, also known as GSHPx, is present in both neurons and glial cells [108, 109]. Overexpression of GPx decreases the amount of neuron loss, reduces hydrogen peroxide accumulation and lipid peroxidation in neurotoxic conditions [110]. Glutathione peroxidase and catalase reduce hydrogen peroxide when acting alone, but more effectively decrease toxicity of exogenous hydrogen peroxide when acting together [111].

### GPX proteins and Parkinson's disease

An immunocytochemical study of GPX1 expression shows that dopaminergic neurons in the SNpc express low levels of this protein, while other regions not affected in PD, such the ventral tegmental area, express higher levels [108]. In addition, GPX1 immunoreactive microglia were reported in samples of SN taken from patients with PD as well as those with Dementia with Lewy Bodies Disease (DLB) [109].

In an experimental model of PD, mice that lack GPX1 under normal oxidative conditions show no apparent neuropathological lesions compared to wild-type mice. However, when challenged with MPTP, a toxin that induces oxidative stress, DA, DOPAC, and HVA levels in these mice are decreased. [112]. Additionally, microarray analysis of mRNA expression in the lateral versus medial tiers of the SN reveals downregulation of *gpx1* and *gpx3* in the lateral tier compared to the medial tier of the SN [113]; this is complimentary to the observation of greater cell loss in the lateral SN of PD patients [114]. Savaskan et al. have shown that GPX4 protein level is increased following a neurodegenerative lesion of the entorhinal cortex. Subsequent immunocytochemical analysis shows that the location of this increase occurs in astrocytes [115]. In cortical samples taken from PD patients, GPX3 and GPX4 protein are also elevated compared to control subjects [116].

In DA neurons of the SN, GPX4 is colocalized with neuromelanin [113]. Examination of brains in SN from PD patients shows that volume-density immunolabeling of GPX4 is reduced; however, relative to the remaining cell density, GPX4 levels are increased compared to control subjects [113]. Further study of Selenoprotein 1 (Sepp1, a transport protein and source of selenium for selenoproteins) and GPX4 in the SN and putamen shows an association between Sepp1 and GPX4 localization in the putamen of control subjects

while the correlation is lost in PD patients. No correlation of Sepp1 and GPX4 immunoreactivity is seen in the SN of PD or control samples [117].

### Glutathione S-Transferases

Glutathione S-Transferases (GSTs) are a class of abundant proteins [118] that function as xenobiotic metabolizing enzymes [119–121] in eukaryotes. This class of enzymes may be viewed as a cellular defense against numerous artificial and naturally occurring environmental agents. GSTs function by catalyzing the conjugation of glutathione to various electrophiles and xenobiotics. Additionally, certain GST's have been shown to have other functions, including modulation of cell survival pathways [122, 123].

In humans, there are three distinct classes of GST's: cytosolic, mitochondrial and microsomal. Within the brain, the cytosolic forms of GST are predominant, and will be the focus here. Each of the cytosolic GSTs is configured with different combinations of monomers that are 199–244 amino acids in length [124, 125]. The ultimate composition of the holoenzyme is critical to the function, since each of the monomers contributes half of the overall GSH:electrophile active site [126, 127]; this dimerization occurs in the presence of increased levels of oxidative stress [128].

At this time, seven classes of cytosolic GST have been identified, and are named alpha, mu, pi, sigma, theta, omega, and zeta [129]. Within each GST molecule, there are two characteristic domains: I and II. Domain I comprises the N-terminal residues (1–80) of the protein whose structure consists of a series of beta pleated sheets and alpha helices. Domain II comprises the remaining residues (81–209±11) and is also referred to as the hydrophobic site (H site) [126]. The GSH binding domain is found in Domain I and is structurally conserved in each of the isoforms [130]. Structural differences among the isoforms are found within domain II. The variable residues in this domain contribute to the array of substrate specificity found among the GSTs [130, 131]. For instance, mu and pi classes contain more polar domain II regions that enable them to react with charged substrates [132] whereas the alpha isoform contains a more hydrophobic domain II [130].

In the brain, the active GSTs are composed of dimers containing alpha, mu, or pi class GST monomers [133–137]. GSTmu is the most highly expressed isoform in the brain in terms of abundance, followed by GSTpi, then GST alpha [135]. Cellular localization studies show that GSTpi and GSTmu are expressed in both neurons and astrocytes [135, 138, 139]. Interestingly, in the SN, the structure most affected in the CNS of PD patients, only GSTpi, but not GSTmu, is found in the A9 DA neurons [135]; a finding that may provide a clue why these neurons are particularly sensitive to oxidative stress. The distribution of GSTs in the brain appears to also be age dependent [136]. GSTpi is the only GST isoform that appears to be expressed in human fetal brain [136, 140], while the alpha, mu and pi classes are found in adult [136]. Unlike other components of the glutathione system, GSTs have also been implicated in signal transduction regulation, specifically in pathways involved in mediation of cell death. Mechanistically, GSTmu has been shown to inhibit apoptosis signal-regulating kinase 1 (ASK1) [141] while GSTpi has been shown to inhibit JNK signaling [128, 142–144].

ASK1, which is activated upon oxidative challenge [145–147], facilitates the downstream activation of JNK and the stress activated protein kinase p38 [148, 149]. GSTmu inhibits ASK1 signaling through a protein-protein interaction [141]. Furthermore, following stress, downstream activation of p38 requires the dissociation of GSTmu from ASK1, suggesting that GSTmu regulates the signal cascade mediated by ASK1 [141, 148].

GSTpi has two distinct functions related to oxidative stress [123]. Under non-stressed conditions, GSTpi exists predominantly in a monomeric form [128]. In this conformation, sequences at the C-terminus of GSTpi have been shown to bind to JNK; which inhibits the interaction of JNK with cJUN and reduces downstream apoptotic signaling [142, 144, 150]. As oxidative stress increases within the cell, GSTpi subunits form dimers. This interferes with the C-terminus interaction with JNK, allowing progression of cell death signaling [128]. The GSH and GST systems collaborate to maintain oxidative homeostasis. When the cellular environment is stress free (i.e. there are low levels of free radicals), GSH levels alone are sufficient to maintain redox balance [151] and monomeric GSTpi bind JNK [128]. As the number of free radicals within the cell increases, GSTpi dimerizes and in this conformation interacts with GSH to more efficiently reduce these free radicals [152], bringing the cell back to homeostasis.

As stated above, GSTpi is the only GST isoform found in the A9 DA neurons [135]; a finding that may provide a clue why these neurons are particularly sensitive to oxidative stress. Suggested by evidence of a decrease in total GSH [20, 153] and increase in oxidative stress in postmortem analysis of PD brains [154–157], alterations in structure or sequence (polymorphisms) of GSTs (particularly GSTmu and GSTpi) may contribute to the disease susceptibility and progression of PD. A number of studies have examined the correlation of GST sequence polymorphisms with PD (Table 1) and with one exception, no associations were found (although see DePalma et al, 1998 [158]).

Although polymorphisms in GSTs alone do not appear to correlate with PD etiology, they do appear to have some effect on other aspects of PD, including age of onset and interactions with environmental agents. For example, Golbe et al reported that age of onset in individuals carrying the A53T alpha-synuclein (PARK1) mutation is positively associated with a GSTpi G-for-A nucleotide substitution at position 313 [159], while McCormick et al identified polymorphisms in GSTpi that correlate with increased risk of PD after exposure to pesticides [160]. Additionally, DePalma et al reported a marked increase in association of PD with a GSTT1\*0 polymorphism when correlated with other PD risk factors, such as rural living and well water consumption [158]. The lack of a consistent correlation between GST polymorphisms and PD does not rule out a significant role for this enzyme in the etiology of PD, as there could be other regulatory influences on these proteins, including those controlling synthesis and breakdown.

### **GSTpi and animal models of Parkinson's Disease**

The role of GSTs in the protection of DA neurons has been examined using several models of Parkinson's disease. Several groups have mutated the Parkin gene in *Drosophila*, and find subtle but significant loss of neurons in the protocerebral posterior lateral (PPL) DA neuron cluster [161] as well as an increase in oxidative stress [162] and a downregulation of GSTO1, which has high thiol transferase activity [163]. Examination of these mutants reveals that when GSTS1 is deleted the subtle loss of DA neurons in the PPL is enhanced. Conversely, overexpression of GSTS1 ameliorates this neurodegeneration [161]. When GSTO1, which has two distinct alleles, GSTO1A and GSTO1B, is deleted, there is an increased sensitivity to the xenobiotic paraquat [162, 164]. The re-expression of the GSTO1A in the null mutant eliminates the sensitivity of the PPL DA neurons to paraquat and suppresses phospho-JNK activity, which is implicated in apoptosis [163].

A number of polymorphisms in the leucine rich repeat kinase 2 (LRRK2) gene have been shown to confer PD in humans [165–167]. The most common polymorphisms in the LRRK2 gene are the G2019S and R1441C alleles, which affect kinase signaling [168] and GTPase activity [169], respectively. Recently, Chen et al have demonstrated that expression of the G2019S polymorphic LRRK2 gene in SH-SY5Y cells induces apoptotic cell death and that

overexpression of GSTP1 reduces this apoptosis. The mechanism for this induction is a G2019S-induced hypermethylation of the GSTP1 promoter that can be reduced by addition of NAC or catalase [170].

Smeyne et al [135] examined the effects of loss of GSTpi on MPP<sup>+</sup> or MPTP-induced dopaminergic neuron death. Using primary cultured dopaminergic cells harvested from the SN of MPTP resistant Swiss-Webster mice [171], inhibition of GST by ethacrynic acid or siRNA directed against GSTpi increases the amount of MPP<sup>+</sup>-induced DA neuron death to levels that mimic neurons from an MPTP-sensitive C57BL/6 strain. Similarly, when MPTP is administered to Swiss-Webster mice carrying a null mutation of GSTpi [172] SNpc DA neuron loss increases to levels seen in the MPTP-sensitive C57Bl/6 strain [135]. Administration of MPTP to mice lacking GSTpi also shows cellular ubiquitination and increased susceptibility to ubiquitin proteasome system (UPS) damage and inactivation [173]. Shi et al [174] demonstrates that overexpression of GSTP1 in Neuro2A cells as well as in primary cortical neurons confers protection from oxidative stress induced by addition of rotenone.

## GSH Transport in the Brain: Multidrug Resistance Proteins (MDRP) and the Blood Brain Barrier (BBB)

Free radicals that have been reduced by thiol conjugation are transported out of the brain through multidrug resistance proteins (MDRP) at the gliovascular interface [175–178]. MDRPs are a family of ATP-binding cassette (ABC) transporters that actively transport electrophilic substances across cell membranes [179]. This large family of proteins (MDR1 (also known as P-glycoprotein), MRP1-MRP6, MRP8, BSEP, and BCRP), is encoded by 10 genes (ABCB1, ABCC1-ABCC6, ABCC11, ABCB11, and ABCG2) [180, 181]. Each member of the ABC transporter family has a unique pattern of expression in tissue, which may vary between and within species [182–184]. In particular, the localization of transporters is specific to the luminal or basolateral membrane of endothelial cells and at astrocytic endfeet of the BBB [181, 183, 185]. In addition to the BBB, these ABC transporters are also found at the blood-cerebrospinal fluid interface, which forms both a physical and enzymatic barrier between the tight ependymal cell junctions of the choroid plexus and cerebrospinal fluid [186, 187], and also expresses proteins critical to modulating ROS detoxification. The transport of free radical conjugates through MDRPs across the BBB is both ATP and sodium dependent [175, 176].

P-glycoprotein (Mdr1), Mrp1, Mrp2 and Mrp5 are the most widely studied of the transporters in the brain. These transmembrane proteins alter their conformation by utilizing ATP to translocate substances, including xenobiotics conjugated with GSH, across membranes [188–193]. Traditionally, it was believed that the expression of these transporters was found solely on capillary endothelial cells [194–196]; however, confocal studies suggest that some of these transporters may be localized to astrocytes [197].

A number of experiments demonstrate that the expression of these efflux transport proteins are correlated to intracellular oxidative stress levels [176, 198–202]. For example, Hong et al [176] demonstrated that BSO depletion of GSH increases P-gp levels, and conversely, treatment with the antioxidant NAC decreases its expression. In hepatocytes, P-gp transports MPP<sup>+</sup> into the cell [203], suggesting that this pore contributes to xenobiotic sensitivity. P-gps can also transport the anti-Parkinsonian drug budipine [204], suggesting that it may also play a protective role in PD. Ziemann et al [198] demonstrated that addition of H<sub>2</sub>O<sub>2</sub> to hepatocytes increases expression of *mdr1*, while Deng et al [205] showed a similar effect on the P-gp protein following genotoxic stress-inducing DNA damage. In models of the BBB, oxidative stress generated by hydrogen peroxide [202] and GSH depletion [176, 201] result



in increased expression of P-gp. Each of these studies demonstrates the cellular coordination of ROS detoxification between GSH or GST regulation and cellular efflux. In conditions of high ROS, where free GSH levels are lower due to binding of electrophiles, MDRP expression is increased. Conversely, when cells are at homeostasis, free GSH levels are elevated and MDRP levels decrease.

MDRPs and GSTs often act in concert to remove free radicals as well as xenobiotic compounds from cells. Nitrogen monoxide (nitric oxide, NO) has been shown to induce iron release from activated macrophages during the cytotoxic defense against tumor cells [206]. Although necessary for cell signaling and enzyme activity, NO and iron are toxic when free and in excess concentrations within the cell [207–209]. Within the cell, free iron can complex with intracellular NO and GSH to form dinitrosyl-dithiol-iron complexes (DNICs) [206, 210]. A recent study has demonstrated the selective relationship between GSTP1, DNIC's and MRP1 [211]. In the presence of NO or iNOS and hyperexpressed MRP1, cells transfected with GSTP1, but not GSTA1 or GSTM1, have reduced iron efflux compared to cells transfected with the control vector. Cells with MRP1 hyperexpression and GSTP1 expression are also resistant to the cytotoxic effects of S-nitroso-glutathione (GSNO), which is a NO generator, demonstrating the critical role for GSTP1 and MRP1 as a mechanism for free radical detoxification. Investigation of DNICs, GSTp, and MRP1 as an effective storage and release mechanism for NO and iron in the SN may be useful to discover the elements at work in the balance of homeostasis versus oxidative stress leading to neurodegeneration.

Numerous studies demonstrate alterations in MDRPs in Parkinson's disease. Expression of MDR1 in blood vessels is reduced in the striatum of PD patients compared to control subjects [212]. Additionally, studies that examined the uptake of [<sup>11</sup>C]-verapamil, a compound that is usually extruded from the brain by P-gp, demonstrate a significant elevation of [<sup>11</sup>C]-verapamil in PD patients compared to controls [213, 214] suggesting that P-gp dysfunction contributes to PD pathogenesis [213, 214]. Polymorphisms in MDRPs have also been examined as they relate to PD risk. Westerlund [215] showed that a 1236C/T, but not 2677G/T/A or 3435C/T increases the risk of PD in a Swedish population. The lack of a direct effect of the 3435T is supported by Funke et al [216] and Furano et al [217]. The 3435T polymorphism, however, is implicated as a contributing risk factor in the development of Parkinson's disease when combined with exposure to pesticides [218, 219].

## GST Expression as a Biomarker for Parkinson's Disease

One of the major obstacles in interrupting the process of pathogenesis in Parkinson's disease is the inability to detect pathology (i.e. SNpc dopaminergic neuron loss, striatal dopamine loss) prior to onset of observable symptoms (tremor). This has led to a search for a presymptomatic Parkinson's disease biomarker. According to the NCI Dictionary of Cancer terms, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease". Numerous functional tests (olfaction, gut motility) as well as imaging modalities (PET, fMRI) have been examined to identify persons with Parkinson's disease prior to symptom onset [220]. However, these are often time reliant on personal narrative or are quite expensive and/or invasive.

Since GSTs are modulated by a number of actions that induce oxidative stress, it is thought that measurement of this class of proteins may allow identification of individuals where this process is aberrant. The observation that certain GSTs are expressed in brain regions affected in Parkinson's disease and change expression in models of PD [135, 139, 174] make these particularly interesting enzymes for study. Werner et al used a proteomic method to examine protein expression in the substantia nigra of PD and age-matched controls and

find increased expression of both GSTmu and GSTpi isoforms in PD patients [221]. Shi et al [174, 222] examined synaptosomal fractions isolated from the frontal lobes of PD patients and find significantly increased levels of GSTpi protein. Maarouf et al [223], also using a proteomic analysis, examined postmortem ventricular cerebrospinal fluid from PD patients and find a significant increase in GSTpi. Recently, Korff et al [224] measured changes in GSTpi protein in blood, comparing GSTpi levels at baseline and at various times after addition of MPP<sup>+</sup>, as an inducer of oxidative stress. They demonstrated that 4 hours after MPP<sup>+</sup>, GSTpi is significantly increased in the white blood cells of PD patients compared to control subjects, while no changes are seen when examining whole blood, plasma or the red cell fraction. These studies suggest that GSTpi and potentially other members of this and other anti-oxidant families may be viable biomarkers for PD.

## GSH and Precursor Delivery as a Therapy for Parkinson's Disease

As discussed earlier, increased levels of oxidative stress and decreased levels of GSH have been described in a number of PD models [69, 73, 74, 76, 77, 80], as well as in the SN of PD patients [6, 19, 20, 85]. Based on these findings, it has been suggested that restoring the level of GSH in brains of Parkinson's disease patients may be a promising therapy to protect the affected DA neurons from further injury [225]. A number of therapeutic compounds have been examined, including GSH alone (via delivery in liposomes and nanoparticles), co-drugs, such as GSH:L-Dopa or GSH:DA conjugates, as well as GSH analogues, and other hybrid compounds. Optimally, successful candidates should be stable during gastrointestinal digestion, undergo bioconversion to constituent compounds that are transported into the brain, navigate to the desired site of action, and protect against the oxidative damage. Additionally, for effective treatment with these GSH analogues, they should be characterized by limited  $\gamma$ -GT metabolism, while also maintaining their reducing ability [226].

Several examples of these types of compounds have been generated and tested. Minimal improvement was reported in studies examining effect of GSH infusion on the amelioration of PD symptoms. Sechi et al [227] used iv infusion of GSH into nine patients with early untreated PD and found that during infusion patients showed a 42% decline in disability compared to vehicle treated controls. This effect lasted 2–4 months after discontinuation of the infusions. However, in a study that examined 21 patients, intravenous administration of GSH produced no significant improvement [228]. Another chemical method has also been used to increase GSH levels in the brain. Lee et al [229] coupled a hydrogen sulfide (H<sub>2</sub>S)-releasing molecule to L-dopa. When injected intravenously, this compound reached the brain and increased dopamine levels by 2.2-fold and GSH by 1.4-fold.

The lack of significant improvement seen following administration of GSH leads to questions regarding the delivery and transport of GSH into the brain. A number of molecules have been used to improve transport through the BBB. Liposomes are artificially prepared vesicles with outer lipid bilayers that encapsulate aqueous materials, including GSH. These liposomes have low toxicity, do not induce an immune reaction and are protected from enzymatic digestion and metabolism [230]. Based on their solubility, liposomes can easily pass through cell membranes including the BBB [231–233]. Although Liposomal-GSH has not been used in human trials, Zeevalk et al show that these conjugates are 100-fold more effective at replenishing GSH levels and preventing degeneration in paraquat + maneb treated mesencephalic cultures than GSH alone. Additionally, the liposomal-GSH is not a substrate for GST's or  $\gamma$ -GT [234].

Nanoparticles provide another method for improving GSH transport into the brain. These are nanometer-scale polymeric substances with a structural and functional organization that

delivers compounds that are encased by, or linked to the surface of the nanoparticle. Nanoparticles provide high capacity drug loading while maintaining resistance to enzymatic degradation, allowing prolonged drug delivery in the plasma with low toxicity side effects [226, 235]. One such nanoparticle, linking GSH and chitosan, maintains improved stability of GSH in oxidative conditions more effectively than free GSH [236]. Another type of nanoparticle used for drug-delivery across the BBB is dendrimers, a tree-like polymer, approximately 5nm in size, to which a number of functional groups can be attached [237]. These dendrimers are used to deliver the anti-inflammatory and anti-oxidant agent N-acetyl-L-cysteine (NAC, a reducing agent like GSH) to LPS stimulated BV-2 microglial cell cultures. The presence of these NAC-containing dendrimers is significantly more effective than NAC alone in reducing H<sub>2</sub>O<sub>2</sub> (68% vs 41%) and TNF- $\alpha$  (77% vs 44%) [238].

Co-drug delivery of compounds, including GSH, links two different compounds that have similar or different modes of action in order to synergize their actions in the brain [239]. A number of these co-drugs have been developed as a potential treatment for Parkinson's disease. The most frequent cofactor for these co-drugs is L-dopa. L-dopa has been conjugated to a number of other agents including entacapone (a COMT inhibitor marketed under the trade name Stalevo®) [240], cysteine [241], N-acetyl cysteine [242], L-Methionine [241], lipoic acid [243], caffeic acid and carnosine [244]. Co-drugs have been made that directly link GSH and L-dopa [245, 246]. Functionally, it is hypothesized that by joining L-dopa to GSH the exogenous GSH can be directed to the specific neurons within the SNpc that are affected in PD. A second application for co-drug development is to better target the protein of interest (in this case GSH) through the BBB, while protecting the protein from enzymatic degradation so that it can be released in a functional state once in the brain. To achieve this goal, More et al developed a series of metabolically stable urea analogues of glutathione [247], that are protected from 3GT cleavage [248].

Other recently generated codrugs include flavanoid compounds that enhance the uptake of cystine/cysteine by uncoupling their uptake from the cystine/glutamate antiporter, X(c)(-). Flavanoids are plant polyphenols with free radical scavenging capacity. Amino acid moieties were added to flavanol compounds to test their effectiveness as neuroprotectants in conditions of glutamate toxicity [249]. Conjugation of the flavanoid epicatechin (EC) with cysteine, cysteamine-EC, increases cell survival and GSH level in a dose-dependent manner [250]. Support for the hypothesis that neuroprotection occurs through increasing cystine/cysteine availability is provided by experiments demonstrating the loss of this effect following BSO treatment or cystine depletion [250].

In addition to co-drugs, glutathione analogues called UPF peptides, each with anti-oxidative capacities, have been synthesized [251]. These compounds link GSH molecules to tyrosine derivatives by an amide bond and have better hydroxyl radical scavenging properties than glutathione alone. Two of these UPF proteins, UPF1 (4-methoxy-L-tyrosinyl- $\gamma$ -L-glutamyl-L-cysteinyl-glycine) and UPF17 (4-methoxy-L-tyrosinyl- $\alpha$ -L-glutamyl-L-cysteinyl-glycine) are shown to increase free radical scavenging by 500-fold [252]. UPF1 is resistant to  $\gamma$ GT activity and increased CuZnSOD activity, suggesting that UPF1 has antioxidant activity independent from GSH [253]. Although not yet tested in animals, or PD patients, these conjugated proteins may provide novel peptide based therapies to slow or ameliorate disease progression.

## Conclusions

The cause of Parkinson's disease is multifactorial, thought to have genetic and environmental etiologies. In each case, the mechanism underlying the disease includes alterations in pathways that increase oxidative stress. In this review, we have discussed the

role of glutathione as well as facets of glutathione metabolism and function, which can be summarized in Figure 2. Further understanding of the mechanisms by which glutathione interacts with free radicals should lead to new approaches for slowing or ameliorating progression of this and other neurodegenerative diseases.

## Abbreviations

|               |   |
|---------------|---|
| <b>ABC</b>    | ATP-binding cassette transporter  |
| <b>ASK1</b>   | apoptosis signal-regulating kinase 1  |
| <b>BBB</b>    | Blood Brain Barrier   |
| <b>BSO</b>    | L-buthionine-(S,R)-sulphoximine   |
| <b>COMT</b>   | Catechol-O-methyltransferase  |
| <b>DA</b>     | Dopamine  |
| <b>DAT</b>    | Dopamine transporter  |
| <b>DHBT-1</b> | 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid |
| <b>DOPAC</b>  | 3,4-Dihydroxyphenylacetic acid  |
| <b>γGCS</b>   | γ-glutamylcysteine synthetase   |
| <b>γGC</b>    | γ-glutamylcysteine  |
| <b>γGT</b>    | γ-glutamyl-n-transpeptidase   |
| <b>GCL</b>    | glutamylcysteine ligase   |
| <b>GPX</b>    | Glutathione Peroxidase  |
| <b>GSH</b>    | Glutathione   |
| <b>GSSG</b>   | glutathione disulfide   |
| <b>GST</b>    | Glutathione S-Transferase   |
| <b>HVA</b>    | Homovanillac Acid   |
| <b>JNK</b>    | c-Jun N-terminal kinase   |
| <b>LDH</b>    | Lactate dehydrogenase   |
| <b>LPS</b>    | Lippopolysaccharide   |
| <b>MDRP</b>   | Multidrug Resistance Protein  |
| <b>MPP+</b>   | 1-methyl-4-phenylpyridinium   |
| <b>MPTP</b>   | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine                                  |
| <b>MTT</b>    | mitochondrial pyruvate-dependent 5,5-Dithiobis (2-nitrobenzoic acid)          |
| <b>NAC</b>    | N-acetylcysteine  |
| <b>OTC</b>    | 2-oxothiadolazine-4-carboxylate   |
| <b>PD</b>     | Parkinson's disease   |
| <b>Pgp</b>    | P-glycoprotein  |
| <b>ROS</b>    | Reactive oxygen species   |
| <b>SIN1</b>   | 3-morpholinopyridone  |

|              |                                   |
|--------------|-----------------------------------|
| <b>SNP</b>   | Single nucleotide polymorphism    |
| <b>SNpc</b>  | Substantia nigra pars compacta    |
| <b>TH</b>    | Tyrosine hydroxylase              |
| <b>VMAT2</b> | Vesicular monoamine transporter 2 |

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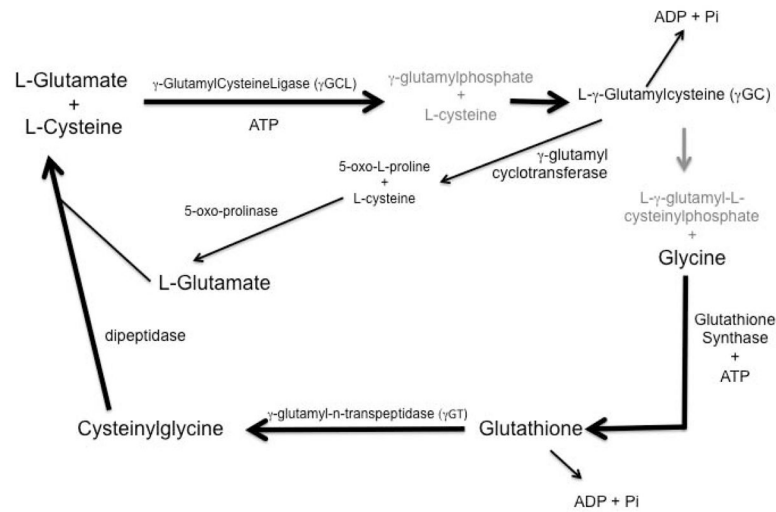
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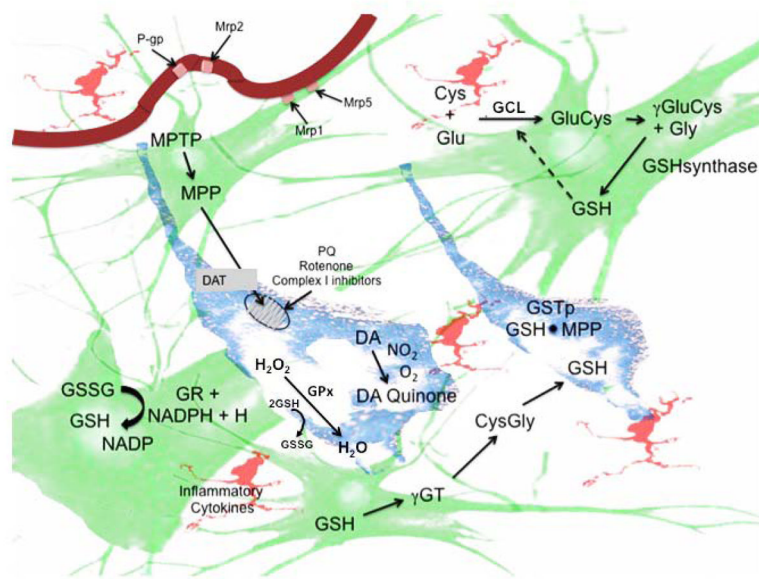
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### Highlights

- Glutathione, a thiol tripeptide, is an important antioxidant in the brain.
- GSH is critical for protecting DA neurons in the SNpc from free radicals damage.
- GPx and GSTs catalyze the reduction of electrophiles using GSH
- GST mutations combined with environmental insults correlate with increased PD risk
- Maintaining GSH levels may provide a therapeutic treatment for PD



**Figure 1.** Glutathione synthesis pathway. Glutathione is synthesized from L-glutamate and L-cysteine in a 2-step reaction catalyzed in an ATP dependent manner by  $\gamma$ -glutamylcysteine ligase (GCL) (also referred to as  $\gamma$ -glutamylcysteine synthetase) and the addition of glycine by glutathione synthase. Glutathione can be recycled to its constitutive amino acids by  $\gamma$ -glutamyl-n-transferase and dipeptidase.



**Figure 2.**

Schematic representation of glutathione synthesis and catabolism in the substantia nigra. Glutathione (GSH) synthesis occurs in astrocytes (green) and dopaminergic (DA) neurons (blue). GSH is synthesized from L-glutamate (Glu) and L-cysteine (Cys) by  $\gamma$ -glutamylcysteine ligase (GCL) and the addition of glycine by glutathione synthase. Once generated, the oxidized form of GSH (GSSG) can be recycled to reduced GSH by glutathione reductase (GR) and NADPH. Additionally, GSH and/or its conjugates can be recycled by  $\gamma$ -glutamyl transpeptidase ( $\gamma$ GT). GSH reduces ROS generated by a number of agents that are transported through the dopamine transporter (DAT), including MPTP, MPP<sup>+</sup>, and rotenone, that block mitochondrial Complex I. GSH can also reduce direct redox agents such as paraquat (PQ) or DA adducts (DA quinone) and inflammatory cytokines released from microglia (pink). GSH maintenance and clearance of conjugated electrophiles requires energy in the form of ATP and NADPH. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is reduced by glutathione peroxidase (GPx) to water using GSH. In DA neurons, the reduction of free radicals is catalyzed by conjugation of GSH to an electrophile by glutathione S-transferase pi (GSTp). Conjugated adducts are transported from the brain parenchyma through MDRPs, including Mrp1 and Mrp5 through the basolateral membrane into capillary endothelial cells. Once in these cells, other MDRPs, including P-gp and Mrp2 transport these to the bloodstream for excretion.

**Table 1**

GST polymorphisms and Parkinson's Disease

| GST isoform   | association with PD          | population  | locus      | Amino acid change   | effect                           | Reference |
|---------------|------------------------------|-------------|------------|---------------------|----------------------------------|-----------|
| <b>GST O1</b> | PD Risk                      | Caucasian   | rs4925     | Ala140Asp           | $p = 0.034$                      | [254]     |
|               | PD Risk                      | Japanese    | rs4925     | Ala140Asp           | none                             | [255]     |
|               | PD Risk                      | Japanese    | rs11191972 | C to T              | none                             | [255]     |
|               | PD Risk with smoking         | California  |            |                     | none                             | [256]     |
|               | PD Risk with smoking         | California  | rs4925     | Ala140Asp           | none                             | [256]     |
|               | PD Risk with smoking         | California  | rs2297235  | Chr10: 10861 A to G | none                             | [256]     |
|               | Age of Onset                 | USA         | rs4925     | Ala140Asp           | none                             | [257]     |
|               | PD Risk                      | Japanese    | rs4925     | Ala140Asp           | none                             | [258]     |
|               | PD Risk,                     |             |            |                     |                                  |           |
|               | Age of Onset                 | Australian  | rs4925     | Ala140Asp           | none                             | [259]     |
|               | PD Risk, Age of Onset        | Australian  | rs4925     | Ala140Asp           | none                             | [259]     |
| <b>GST O2</b> | PD Risk                      | Caucasian   | rs156697   | Asn142Asp           | none                             | [254]     |
|               | PD Risk                      | Japanese    | rs156697   | Asn142Asp           | none                             | [255]     |
|               | PD Risk                      | Japanese    | rs2297235  | -183A to G          | none                             | [255]     |
|               | none                         | California  |            |                     | none                             | [256]     |
|               | PD Risk, Age of Onset        | Australian  | rs156697   | Asn142Asp           | none                             | [259]     |
|               | Age of Onset                 | USA         | rs2297235  | Chr10: 10861 A to G | none                             | [257]     |
| <b>GST M1</b> | PD Risk                      | East Indian | null       | null                | none                             | [260]     |
|               | PD Risk                      | Japanese    | null       | null                | none                             | [255]     |
|               | PD Risk                      | Chilean     | null       | null                | $p = 0.0092$                     | [261]     |
|               | none                         | Portuguese  | null       | null                | none                             | [262]     |
|               | age of onset                 | Greek A53T  | null       | null                | none                             | [159]     |
|               | PD Risk, synergy with CYP2D6 | French      | null       | null                | none                             | [263]     |
|               | PD Risk                      | USA         | null       |                     | none                             | [264]     |
|               | PD Risk                      | Japanese    | null       | null                | none                             | [265]     |
|               | PD Risk, Age of Onset        | Swedish     | null       |                     | $p=.03$ for earlier age of onset | [266]     |
|               | PD Risk                      | UK          | null       |                     | none                             | [267]     |

| GST isoform   | association with PD                     | population  | locus     | Amino acid change      | effect           | Reference |
|---------------|---|-------------|-----------|------------------------|------------------|-----------|
|               | PD Risk with exposure to pesticides     | Australian  | null      |                        | none             | [268]     |
| <b>GST M3</b> | age of onset                            | Greek A53T  | deletion  | intron 6 3-bp deletion |                  | [159]     |
| <b>GST A4</b> | PD Risk                                 | Italian     | mutations | sequence 7 exons       | none             | [269]     |
| <b>GST T1</b> | PD Risk                                 | East Indian | null      | null                   | none             | [260]     |
|               | PD Risk                                 | Japanese    | null      | null                   | none             | [255]     |
|               | none                                    | Japanese    | null      | null                   | none             | [270]     |
|               | age of onset                            | Greek A53T  | null      | null                   |                  | [159]     |
| <b>GST P1</b> | PD Risk                                 | East Indian | rs1695    | Ile105Val              | none             | [260]     |
|               | PD Risk                                 | Japanese    | rs1695    | Ile105Val              | none             | [255]     |
|               | none                                    | Japanese    | rs1695    | Ile104Val              | none             | 18327668  |
|               | PD Risk                                 | Portuguese  | rs1695    | Ile105Val              | odds ratio = 2.0 | [262]     |
|               | PD Risk with smoking                    | California  | rs947894  | Ile105Val              | none             | [256]     |
|               | PD Risk with smoking                    | California  | rs1799811 | Ala114Val              | none             | [256]     |
|               | PD Risk with smoking                    | California  | rs1799811 | Ala114Val              | none             | [256]     |
|               | none                                    | UK          | rs947894  | Ile105Val              | none             | [271]     |
|               | age of onset and exposure to pesticides | UK          | rs1799811 | Ala114Val              | p=.04            | [271]     |
|               | age of onset                            | Greek A53T  | rs1695    | Ile105Val              | p=.02            | [159]     |
|               | age of onset                            | Greek A53T  | rs1799811 | Ala114Val              | p=.05            | [159]     |
|               | PD Risk                                 | USA         |           | Ile104Val              | none             | [264]     |
|               | PD Risk                                 | USA         |           | Ala113Val              | none             | [264]     |
|               | PD Risk and exposure to pesticides      | Australian  | rs1799811 | Ala114Val              | p=0.009          | [268]     |
| <b>GST Z1</b> | age of onset                            | Greek A53T  | Lys32Glu  | Lys32Glu               |                  | [159]     |
|               | age of onset                            | Greek A53T  | Arg42Gly  | Arg42Gly               |                  | [159]     |
|               | PD Risk                                 | Australian  |           | Glu32Lys               | none             | [272]     |
|               | PD Risk                                 | Australian  |           | Gly42Arg               | none             | [272]     |
|               | PD Risk and exposure to pesticides      | Australian  |           | Arg42Gly               | none             | [268]     |