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MINIREVIEW

Glutathione, oxidative stress and neurodegeneration

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There is significant evidence that the pathogenesis of several neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Friedreich's ataxia and amyotrophic lateral sclerosis, may involve the generation of reactive oxygen species and mitochondrial dysfunction. Here, we review the evidence for a disturbance of glutathione homeostasis that may either lead to or result from oxidative stress in neurodegenerative disorders. Glutathione is an important intracellular antioxidant that protects against a variety of different antioxidant species. An important role for glutathione was proposed for the pathogenesis of Parkinson's disease, because a decrease in total glutathione concentrations in the substantia nigra has been observed in preclinical stages, at a time at which other biochemical changes are not yet detectable. Because glutathione does not cross the blood–brain barrier other treatment options to increase brain concentrations of glutathione including glutathione analogs, mimetics or precursors are discussed.

Keywords: Alzheimer's disease; amyotrophic lateral sclerosis; glutathione; neurodegeneration; Parkinson's disease.

The etiology of neuronal death in neurodegenerative diseases remains mysterious. These illnesses are insidious and subtle in onset and run a gradually progressive, inexorable course. They are exemplified by illnesses such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia and cerebellar degeneration. Recent advances in both molecular genetics and neurochemistry have improved our knowledge of the fundamental processes involved in cell death, including oxidative stress and mitochondrial dysfunction. Both processes may interfere with each other.

In this issue, Dringen and colleagues [1] review the potential of the antioxidant glutathione to detoxify reactive oxygen species (ROS) with special emphasis on its metabolism in neurons and glia. In this review we focus on the evidence that oxidative stress is involved in the pathogenesis of neurodegenerative disease and discuss the specific role of glutathione. Of all neurodegenerative diseases, evidence for a dysfunction of glutathione metabolism based on post mortem examinations, animal and cell culture models is strongest in PD.

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Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BSO, buthionine sulfoximine; CSF, cerebral spinal fluid; DOPAC, 3,4-dihydroxybenzoic acid; GSH, glutathione; GSSG, glutathione disulfide (oxidized glutathione); γGT, γ-glutamyltranspeptidase; HNE, 4-hydroxynonenal; H_2O_2, hydrogen peroxide; HVA, homovanillic acid; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $NO^{\cdot}$, nitric oxide; $^{\cdot}OH$, hydroxyl radical; 6-OHDA, 6-hydroxydopamine; 8OH2′dG, 8-hydroxy-2′-deoxyguanosine; PD, Parkinson's disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta.

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INTRACELLULAR SOURCES OF REACTIVE OXYGEN SPECIES

The observation that biomolecules, which consist primarily of carbon, hydrogen, oxygen, nitrogen and sulfur, are disrupted by the presence of oxygen (O_2) is an evolutionary paradox for aerobic life [2]. A wide variety of ROS can be found in biological systems. These ROS differ in their site of formation, their physiological function, their reactivity and their biological half-life. Mitochondria, nitric oxide synthase, arachidonic acid metabolism, xanthine oxidase, monoamine oxidase and P450 enzymes are sources of ROS in the brain. The high metabolic rate of neurons implies a high baseline ROS production. Correspondingly, healthy brain cells possess high concentrations of both enzymatic and small molecule antioxidant defenses (Fig. 1). The enzymes include CuZn-superoxie dismutase and Mn-superoxide dismutase, GSH peroxidase and catalase, as well as the small molecules glutathione, ascorbic acid, vitamin E and a number of dietary flavonoids. Under normal physiological conditions cells thereby cope with the flux of ROS. Oxidative stress describes a condition in which cellular antioxidant defenses are insufficient to keep the levels of ROS below a toxic threshold. This may be either due to excessive production of ROS, loss of antioxidant defenses or both (Fig. 1).

OXIDATIVE STRESS AND THE SHAPE OF CELL DEATH

Loss of glutathione and oxidative damage have been suggested to constitute early, possibly signaling events in apoptotic cell death [3,4]. In thymocytes, a decrease of GSH and disruption of the mitochondrial transmembrane potential preceded the onset of apoptosis [5,6]. A rapid loss of GSH was found recently in IL3 withdrawal-induced apoptosis of FL5.12 cells. Efflux of GSH constitutes a step of Fas-induced apoptosis [7,8].

Strong evidence that glutathione depletion causes cell death comes from cell culture studies by Li and colleagues [9]. Using

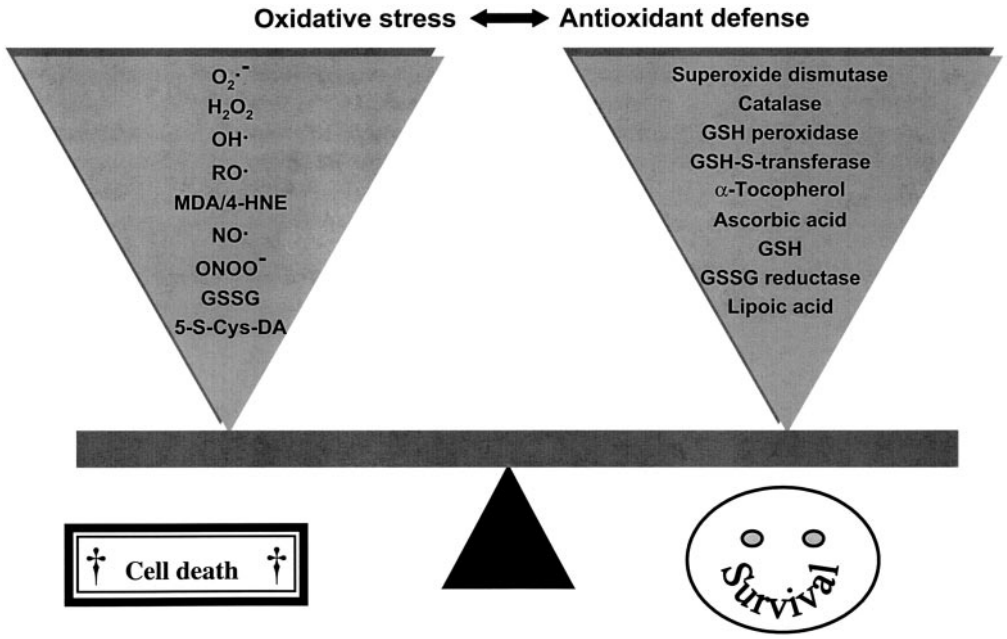

Fig. 1. Balance of ROS generation and antioxidative systems. An imbalance of both systems due to either excessive production of ROS (left) or reduced antioxidant defense (right) leads to oxidative stress.

immature cortical neurons and a neuronal cell line, they showed that a decrease in GSH triggers the activation of neuronal 12-lipoxygenase which leads to the production of peroxides, the influx of Ca^{2+} and ultimately cell death. Along the same lines, using cerebellar granule neurons and PC12 cells, we recently showed that direct depletion of mitochondrial and cytoplasmic GSH resulted in increased generation of ROS, disruption of the mitochondrial transmembrane potential and rapid loss of mitochondrial function [10,11]. In contrast, during inhibition of GSH synthesis, mitochondrial GSH was relatively maintained, no ROS were detected and the mitochondrial transmembrane potential was unchanged. Although cell death could be blocked by protein synthesis inhibitors, suggesting an active type of cell death, dying cells did not display features of apoptosis, indicating that although loss of GSH has been reported in a number of paradigms of apoptotic cell death, loss of GSH per se does not necessarily result in apoptotic cell death.

GLUTATHIONE AND AGING

The most reliable and robust risk factor for neurodegenerative diseases is normal aging. There is substantial evidence that mitochondrial function declines with age. Direct evidence for age-dependent increase in oxidative damage to mitochondrial DNA comes from measurements of 8-hydroxy-2′-deoxyguanosine (8OH2′dG), which is an oxidized form of deoxyguanosine that occurs following attack by a variety of free radicals [12]. Therefore, antioxidant defenses may play a major role during normal aging.

An age-related decline in GSH has been observed in a number of senescent organisms including mosquitoes, adult houseflies, fruit flies, mice, rats and humans [13]. Glutathione concentrations in the cerebral spinal fluid (CSF) of humans decrease during aging [14]. Enforced ectopic expression of GSH prolongs the life span [13]. Such studies suggest that age-related decreases in GSH may represent a key factor in the aging process and may underlie a number of changes occurring in normal aging and the onset of various diseases.

GLUTATHIONE IN PARKINSON'S DISEASE

Pathologically, the hallmark of idiopathic Parkinson's disease (PD) is loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to the major clinical and pharmacological abnormalities that characterize the disease. The cause of neuronal loss in SNpc is not known. However, recent advances have been made in defining morphological and biochemical events in the pathogenesis of the disease. Inhibition of oxidative phosphorylation, excitotoxicity and generation of ROS are considered important mediators of neuronal death in PD [15].

Oxidative stress

The concept that oxidative stress occurs in PD derives primarily from the fact that the metabolism of dopamine, by chemical or enzymatic means, can generate free radicals and other ROS (Table 1). Auto-oxidation of dopamine leads to the formation of neuromelanin and can generate quinone and semiquinone species and other ROS. Perhaps more importantly, enzymatic oxidation of dopamine catalyzed by monoamine oxidase leads to the formation of hydrogen peroxide (H_2O_2) as well as its deaminated metabolites 3,4-dihydroxybenzoic acid (DOPAC) and homovanillic acid (HVA). Normally, H_2O_2 is inactivated by catalase or by glutathione peroxidase in a reaction in which GSH is used as a cosubstrate. Because catalase is compartmentalized into peroxisomes the detoxification of cytosolic and mitochondrial peroxides depends predominantly on glutathione peroxidase. H_2O_2 can react with Fe^{2+} and forms the highly reactive and cytotoxic hydroxyl radical ($^{\cdot}OH$) via the Fenton reaction. The situation is likely to be self-perpetuating because dopamine depletion caused by a decrease in dopamine neurons leads to a compensatory increase in dopamine turnover, with increased formation of H_2O_2 and increased demands on the glutathione system in the remaining neurons. This hypothesis is supported by experimental studies demonstrating that enhanced

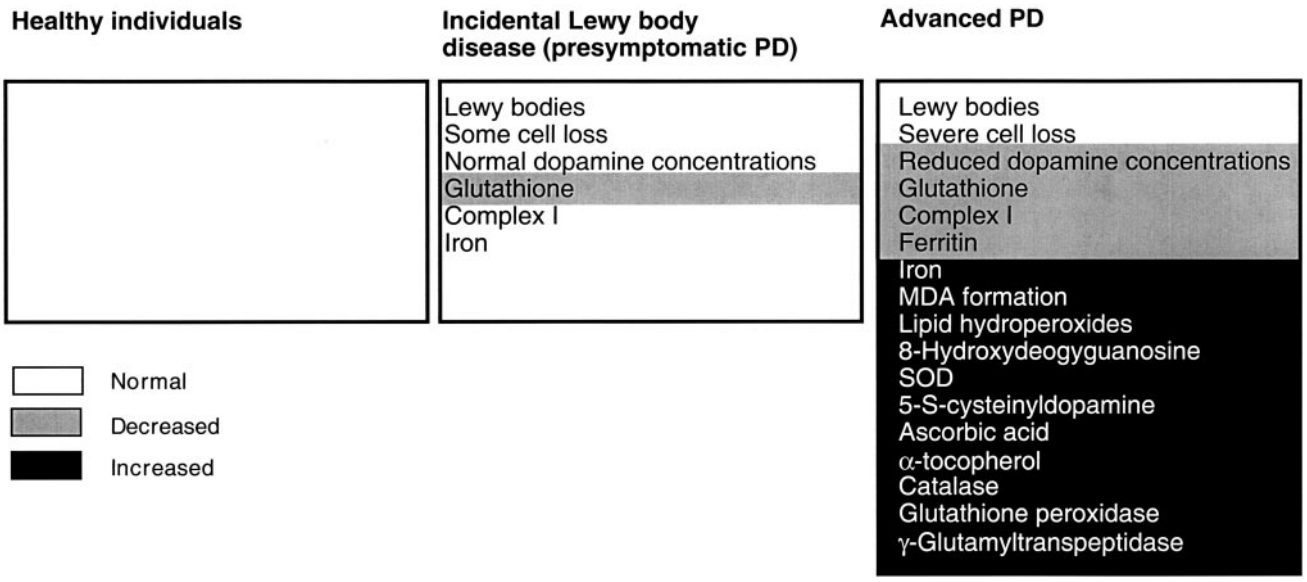

Fig. 2. Evidence for progressive oxidative stress in the pathogenesis of Parkison's disease. In advanced, clinically detectable Parkinson's disease different biochemical alterations occur as depicted in the right column. Of these only a loss of total glutathione is detectable in presymptomatic Parkinson's disease, and this appears to be an early event in the pathogenesis of PD. Modified from Jenner [76]

dopamine turnover is associated with increased formation of oxidized glutathione (GSSG) which, in turn, can be prevented by inhibitors of dopamine metabolism [16]. A variety of critical biomolecules, including lipids, proteins and DNA, can be damaged by ROS, thereby potentially leading to neurodegeneration.

Depletion of GSH

Oxidative stress may be initiated by a decline in the anti-oxidative defense system or oxidative stress caused by other factors may decrease the concentrations of antioxidants. Alterations of antioxidant defenses support the hypothesis that oxidative stress may play an important role in the pathophysiology of PD. The most robust and significant alteration in the antioxidant defense is a decrease in GSH concentration. Initially, a complete absence of GSH in the presence of high GSSG concentrations was reported [17–19]. However, these studies were criticized for methodological reasons, suggesting post mortem artifacts or inappropriate analytical techniques [20]. More recently, total glutathione levels were reported to be decreased in SNpc and the degree of disease severity correlated with the extent of GSH loss [21]. Although a decrease in GSH concentrations of 30–40% was detected, there was no corresponding increase in the level of GSSG [22,23]. These results are specific for SNpc of PD patients, because GSH levels are not reduced in other brain areas in PD patients or in patients with other neurodegenerative diseases that also affect dopaminergic neurons. GSH is decreased to almost the same degree in patients with incidental Lewy body disease, that is considered a preclinical, still asymptomatic form of PD. At that time point other biochemical markers that are altered later during the disease, e.g. the activity of complex I of the electron transport chain, are still normal [24] arguing for an important and early role of GSH in the pathogenesis of PD (Fig. 2). However, it remains unclear whether GSH is depleted in the cytosol or/and in mitochondria. Although GSH tends to be conserved preferentially in mitochondria, the concentration of GSH in mitochondria may be most relevant for the disposal of ROS and cell fate [10,11,25]. In PD patients reduced immunostaining of GSH is observed in dopaminergic neurons [26]. However, in the brain most of the GSH is localized in glia [27,28]. To account for a 40% decrease in GSH concentration in SNpc of PD, GSH is probably decreased not only decreased in dopaminergic neurons, which only make up 1–2% of the total cell population, but also in glial cells. Depletion of glial GSH is critical for the survival of dopaminergic neurons in culture [29,30]. Reasons may include glial production of NO and cytokines [30] or an insufficient supply of GSH precursors to neurons by astrocytes [1].

Insights into the pathogenesis of PD have been achieved experimentally using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP produces irreversible clinical, biochemical and neuropathological effects which closely mimic those observed in idiopathic PD [31]. This meperidine analog is metabolized to 1-methyl-4-phenylpyridinium (MPP^+) by the

Table 1. Production of ROS with specific emphasis on PD. Mao, monoamine oxidase. SOD, superoxide dismutase. Reactions that result in the generation of ROS: Generation of (1,3) superoxide ($^{\cdot}O_2^-$); (2,4,10) a semiquinone radical; (4,5,6,7) hydrogen peroxide (H_2O_2) (8,11) hydroxyl radicals ($^{\cdot}OH$), or (11) peroxynitrite (ONOOH).

$O_2 + 1e^- \rightarrow {}^{\cdot}O_2^-$

(2) Quinone + $^{\cdot}O_2^-$ + H^+ → Semiquinone + O_2

(3) Semiquinone + O_2 → Quinone + $^{\cdot}O_2^-$ + H^+

(4) Catecholamine + $^{\cdot}O_2^-$ + H^+ → Semiquinone + H_2O_2 (MAO)

(5) R-CH_2NH_2 + H^+ + O_2 + H_2O → R-CHO + NH_4^+ + H_2O_2 (SOD)

(6) O_2^- + $^{\cdot}O_2^-$ + $2H^+$ → H_2O_2 + O_2

(7) Fe^{2+} + $^{\cdot}O_2^-$ + $2H^+$ → Fe^{3+} + H_2O_2

(8) Fe^{2+} + H_2O_2 → Fe^{3+} $^{\cdot}OH$ + OH^-

(9) Fe^{3+} + $^{\cdot}O_2^-$ → Fe^{2+} + O_2

(10) Fe^{3+} + Catecholamine → Fe^{2+} + Semiquinone

(11) O_2^- + $NO^{\cdot}$ → ONNOH → $^{\cdot}OH$ + $ONO^{\cdot}$

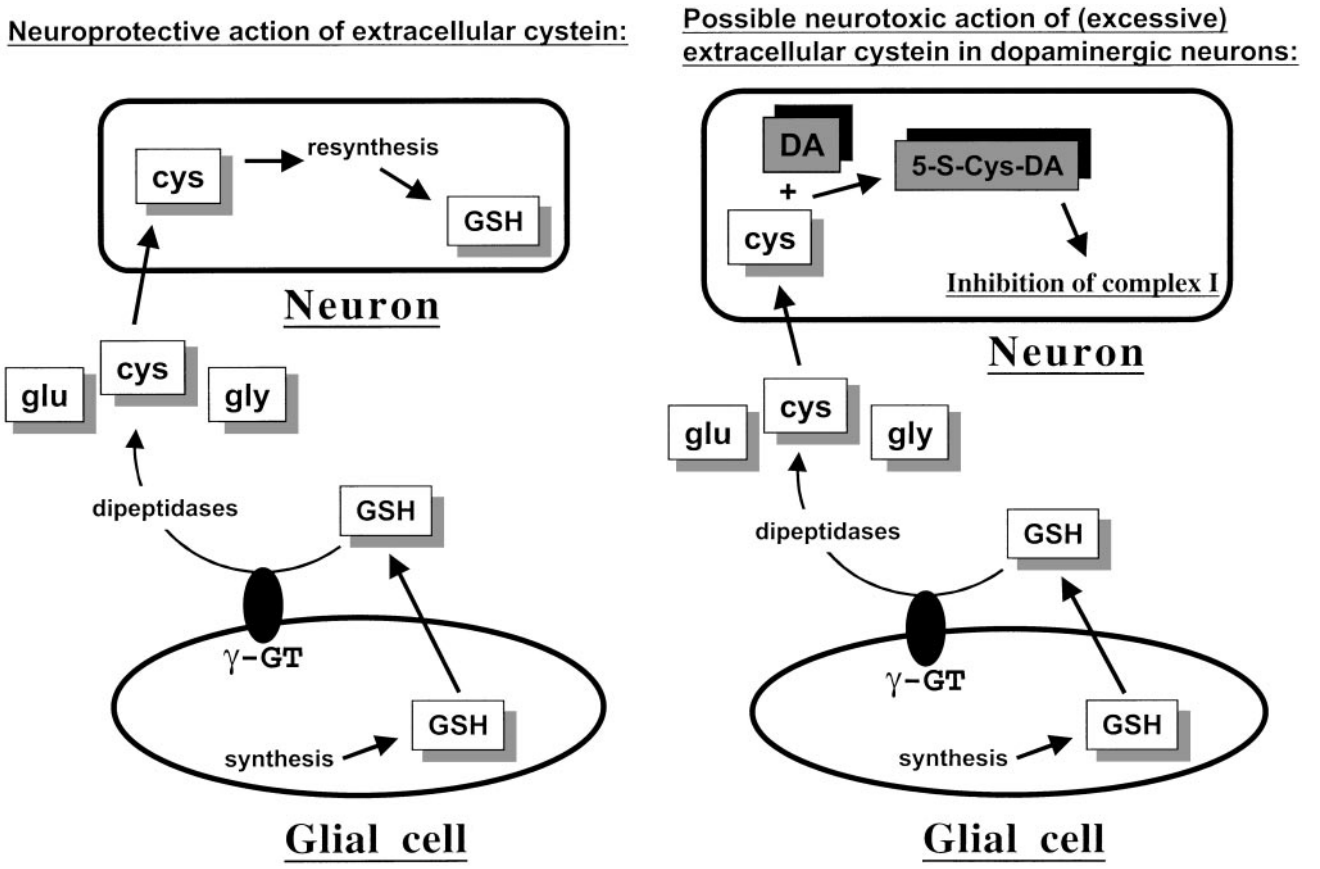

Fig. 3. Hypothesized mechanisms for GSH and its metabolites in dopaminergic neurons. The uptake of the GSH metabolite cysteine may lead to neuroprotection (left) or neurodegeneration (right), depending on whether GSH or 5-*S*-cysteinyldopamine (5-S-Cys-DA) is formed in dopaminergic cells.

enzyme monoamine oxidase B. MPP^+ is subsequently selectively taken up by dopaminergic terminals and concentrated in neuronal mitochondria in the substantia nigra. MPP^+ binds to and inhibits complex I of the mitochondrial electron transport chain [32], thereby producing the same biochemical defect as detected in SNpc of PD patients.

In animals, a chronic, experimentally induced decrease in GSH concentration in the brain does not lead directly to reduced viability of dopaminergic neurons in SNpc or to a decrease in the number of dopaminergic terminals in the striatum [33], but increases the susceptibility of dopaminergic neurons against specific neurotoxins. The degree of striatal dopamine depletion and the loss of nigral tyrosine hydroxylase-immune responsive cells produced by 6-hydroxydopamine (6-OHDA) or MPTP/MPP^+ is enhanced by prior depletion of GSH with buthionine sulfoximine (BSO) administration [34–36]. The importance of the glutathione system in MPTP toxicity was further studied using GSH peroxidase-deficient mice [37]. In these mice, administration of MPTP resulted in greater depletions of dopamime, DOPAC and HVA than those seen in wild-type control mice. That GSH peroxidase may play an important role is further supported by studies showing that excitotoxic lesions induce glutathione peroxidase in activated microglia [38].

The reason for GSH depletion in PD is not clear. Because there is no corresponding increase in GSSG it may not be explained solely by oxidative stress. However, there is no failure of GSH synthesis, because the activity of γ-glutamylcysteine synthetase in the substantia nigra is normal in PD [22]. Interestingly, there is an increase in the activity of γ-glutamyltranspeptidase (γGT) in PD [22]. γGT is a membranous ectoenzyme that catalyzes the transfer of the γ-glutamyl moiety from GSH or a glutathione conjugate onto an acceptor molecule [1]. Extracellular GSH serves as a substrate for the astroglial ectoenzyme γGT. The product of the γGT reaction, the dipeptide cysteinylglycine, is hydrolyzed to cysteine and glycine, which can be taken up and used by neurons in the synthesis of GSH (Fig. 3, left). Because neurons cannot take up GSH directly, an increase in γGT activity may reflect a compensatory up-regulation to provide dipeptide precursors for neurons to generate more GSH. Alternatively, the release of GSH from nigral glia and the increased activity of γGT may be the initial step in the pathogenesis of PD (Fig. 3, right). If cysteine is not used for the synthesis of glutathione, it may react with the dopamine-*o*-quinone, which results from non-enzymatic oxidation of dopamine, to form 5-*S*-cysteinyldopamine [39]. In the substantia nigra of PD patients cysteinyl adducts of 3,4-dihydroxyphenylalanine (L-Dopa), dopamine and DOPAC are increased compared with controls. Importantly, these conjugates can be subsequently converted to highly cytotoxic dihydrobenzothiazine derivates, which may act as irreversible inhibitors of complex I [40–42].

Glutathione transferases

Glutathione transferases are an ubiquitous group of detoxification enzymes involved in the metabolism of pesticides and other toxins. Glutathione transferases have direct antioxidant activity and are involved in the metabolism of dopamine. The activity of glutathione transferase has been reported to be

normal in the brains of PD patients. Although there is no association between idiopathic PD and glutathione transferase polymorphism, the distribution of the glutathione transferase 1 genotype differed significantly between patients and controls who had been exposed to pesticides [43]. Therefore, glutathione transferase 1, which is expressed in the blood–brain barrier, may influence response to neurotoxins and may explain the susceptibility of some people to the parkinsonism-inducing effects of pesticides, however, it is unlikely to explain the decrease of GSH concentrations in SNpc of PD patients.

Defect of oxidative phosphorylation

Another consistent finding in PD patients is a defect in oxidative phosphorylation, in particular, a selective and specific decrease in complex I activity of the electron transport chain in the substantia nigra [44]. It remians controversial whether a decrease in GSH precedes the defect of oxidative phosphorylation or vice versa. Studies on patients with incidental Lewy body disease suggest that a decrease in GSH concentrations may precede the impairment of oxidative phosphorylation (Fig. 2) [24]. Depletion of mitochondrial GSH but not cytosolic GSH in PC12 cells results in the generation of ROS and inhibition of oxidative phosphorylation [11]. Furthermore, inhibition of complex I using MPP^+ does not lead to the generation of ROS directly but affects glutathione homeostasis [45]. In contrast, depletion of GSH by BSO produces enlargement and degeneration of mitochondria in neonatal rats [25] and a decrease in the activity of complexes I and IV in weaning rats [46], arguing for a sequence of primary disturbance of glutathione homeostasis and secondary inhibition of oxidative phosphorylation in the pathogenesis of PD.

GLUTATHIONE IN ALZHEIMER'S DISEASE

Strong evidence that oxidative stress is involved in the pathogenesis of AD comes from a clinical study showing that oral vitamin E intake delayed progression in patients with moderately severe impairment from AD [47]. A role of oxidative stress in AD is further supported by increased levels of thiobarbituric acid-reactive substances, a measure of lipid peroxidation [48,49]. However, studies reporting a disturbance of glutathione homeostasis are less clear. The total brain levels of GSH appeared to be unaffected in AD [50], whereas GSH peroxidase and GSSG reductase were found to be elevated in different brain regions [48] or unchanged [49]. Transcription of GSH peroxidase and GSSG reductase was elevated in hippocampus and inferior parietal lobule, but not in cerebellum of AD patients, which may reflect the protective gene response to the increased peroxidation in the brain regions showing severe AD pathology [51]. 4-Hydroxynonenal (HNE), one marker of lipid peroxidation, is neurotoxic in neuronal culture and *in vivo* and is elevated in AD brain and CSF. The levels of glutathione transferase, a protective enzyme against aldehydes and especially HNE were decreased in the brain and ventricular CSF of autopsied AD and normal control subjects [52]. Together these data imply that oxidative stress plays an important role in the pathogenic process but that alterations in the glutathione system are secondary to other events leading to neurodegeneration.

GLUTATHIONE IN AMYOTROPHIC LATERAL SCLEROSIS

The discovery that some families with autosomal-dominant ALS have mutations in the CuZn superoxide dismutase gene has suggested that oxidative stress may play an important role in the pathogenesis of ALS [53]. Animal studies have shown that transgenic expression of mutated superoxide dismutase leads to an ALS phenotype despite normal or increased superoxide dismutase activity [54]. Although mutations in the superoxide dismutase gene may not lead directly to the generation of ROS by a failure to detoxify superoxide, the gain of function mutations are likely to cause oxidative stress, e.g. by the formation of peroxynitrite. 3-Nitrotyrosine, a marker of peroxynitrite formation, is increased in cortex, spinal cord and CSF of patients with autosomal dominant or sporadic ALS, as well as in transgenic animals [55–57]. In addition, other markers of oxidative damage, e.g. protein carbonyl and nuclear DNA 8OH2′dG levels are increased in the motor cortex of sporadic ALS patients [53]. Furthermore, increased modification of proteins by HNE was found in the lumbar spinal cord of ALS patients vs. that of neurologically normal controls [58]. HNE concentrations were increased in the CSF of ALS patients [59].

Although the evidence for oxidative stress is strong in ALS, the role of altered glutathione metabolism is less clear. Because there is strong evidence that elevated extracellular levels of glutamate in ALS cause excitotoxicity and contribute to cell death of motoneurons, alterations of glutathione metabolism would not only point to enhanced generation of ROS but, in the case of increased GSH hydrolysis by γGT, also implicate increased concentrations of glutamate. However, there are no convincing reports of GSH concentrations in the cortex or spinal cord of ALS patients. The increase of GSH-binding sites in the spinal cord of ALS patients [60,61] may be interpreted as an up-regulation caused by a deficiency of GSH. Results of studies on GSH peroxidase activity in ALS are controversial. Reduced GSH peroxidase activities in red blood cells of sporadic ALS patients were reported in some studies [62,63], but others found this association only when patients were treated with insulin-like growth factor-1 [64]. In spinal cord or cerebral cortex of ALS patients either no detectable [65], reduced [66] or normal GSH peroxidase activities were found [67].

GLUTATHIONE IN SCHIZOPHRENIA

A 27% decrease in GSH has been reported in the CSF of drug-free schizophrenic patients [68]. In contrast, concentrations of dopamine, serotonin and their metabolites were not different from controls. These results were confirmed by *in vivo* proton magnetic resonance spectroscopy which showed a 52% decrease in GSH in the frontal cortex of schizophrenic patients compared with controls. The authors argue that a primary decrease of GSH may explain both the glutamate hypofunction and the dopamine dysfunction hypothesis.

THERAPEUTIC APPROACHES FOR NEURODEGENERATIVE DISEASES

If alterations in glutathione metabolism play an important role in the pathogenesis of the neurodegenerative diseases discussed, treatments that lead to enhanced synthesis of GSH or that inhibit its degradation may result in a slowing of disease progression. Because GSH itself penetrates the blood–brain barrier only poorly and cannot be taken up by neurons directly, treatments with GSH monoethyl ester, glutathione precursors or other glutathione analogs have been used in patients or animal models.

The GSH analog YM 737 provides protection against cerebral ischemia in rats by inhibiting lipid peroxidation [69].

Because glutathione synthesis in neurons is limited by the availability of cysteine [1], compounds that can be metabolized to cysteine could be used as pro-drugs to increase neuronal GSH concentrations. In the murine mutant wobbler, treatment with the glutathione precursor *N*-acetyl-L-cysteine resulted in a significant reduction of motor neuron loss and elevated glutathione peroxidase levels within the cervical spinal cord [70]. Treatment with L-2-oxothiazolidine-4-carboxylate, a cysteine precursor, stimulates growth and normalizes tissue glutathione concentrations in rats fed a sulfur amino acid deficient diet [71]. However, L-2-oxothiazolidine-4-carboxylate is only converted to cysteine by cells expressing 5-oxoprolinase. Because cultured neurons are not able to use L-2-oxothiazolidine-4-carboxylate as a GSH precursor [72] the observed increase of brain GSH may be due to metabolism in glial cells. Unfortunately, the therapeutic window for treatment with substances that increase brain cysteine may be narrow, because cysteine is potentially toxic for neurons [73]. Alternatively, GSH in brain can be increased by intracerebroventricular administration of the dipeptide γ-glutamylcysteine [74]. Neurons can utilize either γ-glutamylcysteine or cysteinylglycine for the synthesis of GSH [75].

In 13 subjects with ALS, the safety and pharmacokinetic properties of procysteine, a cysteine prodrug that increases levels of intracellular glutathione was found. Procysteine entered the CSF after both intravenous and oral dosing and accumulated to significant levels in CSF [14]. However, no increase of CSF glutathione concentrations were found at 4 h after i.v. infusion of procysteine.

Although a disturbance of glutathione homeostasis has been implicated in the pathogenesis of several neurodegenerative diseases it remains open to debate whether: at least in some illnesses, this is a primary defect or only a consequence of ROS generation; brain glutathione can be increased safely using different treatment strategies; and an increase of brain glutathione will result in clinical benefit and/or neuroprotection in animal models or in human diseases.

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