Published in final edited form as:

Neurochem Int. 2007 March ; 50(4): 671–680.

Altered glutathione homeostasis in animals prenatally exposed to lipopolysaccharide

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Abstract

We previously reported that injection of bacterial lipopolysaccharide (LPS) into gravid female rats at embryonic day 10.5 resulted in a birth of offspring with fewer than normal dopamine (DA) neurons along with innate immunity dysfunction and many characteristics seen in Parkinson's disease (PD) patients. The LPS-exposed animals were also more susceptible to secondary toxin exposure as indicated by an accelerated DA neuron loss. Glutathione (GSH) is an important antioxidant in the brain. A disturbance in glutathione homeostasis has been proposed for the pathogenesis of PD. In this study, animals prenatally exposed to LPS were studied along with an acute intranigral LPS injection model for the status of glutathione homeostasis, lipid peroxidation, and related enzyme activities. Both prenatal LPS exposure and acute LPS injection produced a significant GSH reduction and increase in oxidized GSH (GSSG) and lipid peroxide (LPO) production. Activity of γ -glutamylcysteine synthetase (GCS), the rate-limiting enzyme in *de novo* GSH synthesis, was up-regulated in acute supranigral LPS model but was reduced in the prenatal LPS model. The GCS light subunit protein expression was also down-regulated in prenatal LPS model. GSH redox recycling enzyme activities (glutathione peroxidase, GPx and glutathione reductase, GR) and glutathione S-transferase (GST), γ -glutamyl transpeptidase (γ -GT) activities were all increased in prenatal LPS model. Prenatal LPS exposure and aging synergized in GSH level and GSH related enzyme activities except for those (GR, GST, and γ -GT) with significant regional variations. Additionally, prenatal LPS exposure produced a reduction of DA neuron count in the substantia nigra (SN). These results suggest that prenatal LPS exposure may cause glutathione homeostasis disturbance in offspring brain and render DA neurons susceptible to the secondary neurotoxin insult.

Keywords

Parkinson's disease; Lipopolysaccharide; Glutathione; Prenatal; Dopamine

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1. Introduction

Recent studies have demonstrated that inflammation induced by intranigral injection of lipopolysaccharide (LPS) led to selective loss of nigral dopamine (DA) neurons and extensive activation of microglia (Castano et al. 1998; Herrera et al. 2000; Gao et al. 2002; Arimoto and Bing 2003). Our previous studies showed that rat fetuses exposed to bacterial lipopolysaccharide (LPS) at embryonic day 10.5 (E10.5) resulted in a birth of animals with fewer than normal DA neurons (Ling et al. 2002) along with many other characteristics seen in Parkinson's disease (PD). These suggest that prenatal LPS exposure can potentially model the PD. One striking feature of prenatal LPS PD model is the synergistic effect of prenatal LPS exposure and postnatal toxin exposure on DA neuron toxicity. Rats prenatally exposed to LPS showed increased sensitivity to intra-jugular low dose rotenone infusion (Ling et al. 2004a) or supranigral infusion of low dose LPS (Ling et al. 2006). These animals lost greater amount of DA neurons and had significant increases in number of reactive microglia and levels of proinflammatory cytokine tumor necrosis factor alpha (TNF- α) in substantia nigra (SN). However, the mechanisms underlying such differences were unidentified.

Glutathione (GSH) is an important soluble antioxidant present at high concentrations in the brain (Dringen et al. 2000). Postmortem study has shown that there was a 40% decrease in reduced form of glutathione (GSH) in SN of PD patients (Sian et al. 1994). In addition, it was proposed that GSH depletion is the first indicator of oxidative stress during PD process (Bharath et al. 2002). *In vitro* study has shown that LPS treatment decreased GSH levels in astrocytes in mesencephalic cultures in a dose-dependent manner (Bharath et al. 2002). This suggests that LPS insult may cause GSH depletion in glial cells that may contribute to the increased DA neuron loss seen in the prenatal LPS PD model.

Therefore, the present study set out to assess the effects of prenatal LPS exposure on glutathione metabolism in rat brain. The major enzyme activities in *de novo* GSH synthesis, redox cycling, and glutathione utilizations were also assessed and compared with an acute model using supranigral LPS administration. The results in the current study highlight the altered glutathione homeostasis in the animals prenatally exposed to LPS, which may contribute to the increased susceptibility of these animals to a secondary neurotoxin insult.

2. Experimental procedures

2.1 Prenatal LPS treatment

Timed-gravid female Sprague-Dawley (SD) rats (Zivic-Miller, Allison Park, PA) were maintained in an environmentally regulated animal facility for the duration of the study. At embryonic day 10.5, each gravid female received a single injection (i.p.) of either LPS (Sigma, St. Louis, MO; serotype *Escherichia coli* 026:B6; L-8274; 10,000 endotoxin units/kg) or saline. Dose and time was chosen according to our previous study (Ling et al. 2002). Animals were allowed to deliver normally. Pups were weaned after 21 days. Then one male pup from each litter was chosen and randomly assigned to one of eight groups (n=5 per age/prenatal treatment group). After 4 or 17 months, the rats were anaesthetized using sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with ice-cold saline. Then the brains were quickly removed and frozen in 2-methylbutane and stored in -80°C for less than 2 months for biochemical assessments. For immunohistochemistry staining, the littermates were perfused with ice-cold saline followed by perfusion with Zamboni's fixative (7.5% saturated picric acid, 12 mM NaH₂PO₄, 88 mM NaHPO₄, and 4% paraformaldehyde). The brains were further fixed for 24 hr after removal from skull and processed for tyrosine hydroxylase (TH) immunohistochemistry. All animal protocols and procedures used in these studies were approved by the Institutional Animal Care and Utilization Committee (IACUC) of Rush University Medical Center.

2.2 Supranigral LPS injection

Animals for supranigral LPS injection were not the littermates of animals receiving prenatal treatment. Male SD rats at 4 months of age received unilateral supranigral injection of LPS (10 µg/4 µl in saline) or saline at the following coordinates: anterior/posterior = -4.8 mm, medial/lateral = -2.0 mm, dorsal/ventral = -7.6 mm (n=5 per treatment group). This corresponded to the largest segment of SN in the coronal plane. The dose was chosen according to other report (Iravani et al. 2005). Three days after LPS injection, the rats were anesthetized with pentobarbital and sacrificed. The brains were processed as described above for biochemical assessments.

2.3 Preparation of brain homogenates

The brains were dissected while still frozen as previously described (Ling et al. 2004a). The striatum, midbrain, cerebral cortex, and cerebellum were harvested and weighted. For postnatally LPS-injected rats, only midbrain was harvested. The tissues were homogenized with an ultrasonic dismembrator (Biologics Inc, Gainesville, Virginia) for 12 rapid pulses in ice-cold 0.1M phosphate-buffered saline (PBS, pH 7.4) containing 0.1mM EDTA. Then the homogenates were centrifuged at $14,000 \times g$ for 30 min at 4°C and the supernatants were collected for different analyses. Protein concentrations were determined by Bio-Rad (Hercules, CA, USA) DC protein assay kit.

2.4 Glutathione measurement

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured by a commercial kit supplied by Cayman Chemical (Ann Arbor, MI) according to the method of Griffith (Griffith 1980) and Tietze (Tietze 1969). Briefly, 100 µl of supernatant of brain homogenate was added to an equal volume of the metaphosphoric acid and then centrifuged at $2,000 \times g$ for 2 min to remove protein. Then 50 µl of 4 M triethanolamine per ml of the supernatant was added to increase the pH of the sample. For total GSH assay, 50 µl of sample was added to 150 µl of a reaction mixture containing 0.4 M 2-(N-morpholino) ethanesulphonic acid, 0.1M phosphate (pH 6.0), 2 mM EDTA, 0.24 mM NADPH, 0.1 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), and 0.1 unit glutathione reductase (GR). The reaction was carried out at 37 °C for 25 min and then total glutathione was determined by absorbance at 412 nm using GSSG as standard. For the measurement of GSSG, GSH was removed from the reaction by adding 10 µl of 1 M 2-vinylpyridine solution per ml of supernatant. Then GSSG remained in the reaction was quantified as total GSH assay. The amount of reduced GSH was obtained by subtracting GSSG from total glutathione. Each assay was performed in duplicate and GSH and GSSG were expressed as µmol/g tissue.

2.5 Lipid peroxide (LPO) measurement

Lipid peroxidation results in a formation of LPO that can therefore be used to indicate the levels of lipid peroxidation in tissue. LPO was quantified with Cayman LPO assay kit by a method of Mihaljevic et al (Mihaljevic et al. 1996). Briefly, 100 µl of supernatant of brain homogenate was added to an equal volume of saturated methanol and 1 ml of cold chloroform. The mixture was mixed thoroughly, and then centrifuged at $2,000 \times g$ for 5 min to extract LPO into chloroform layer. Then 500 µl of chloroform extract of sample was mixed with 450 µl chloroform-methanol solvent and 50 µl of freshly prepared chromogen (containing 4.5 mM ferrous sulfate in 0.2 M hydrochloric acid) in glass tube and measured the absorbance at 500 nm after 5 min incubation. The absorbances obtained from known concentrations of LPO were used to construct a standard curve. Each assay was performed in duplicate and LPO was expressed as nmol/g tissue.

2.6 γ -Glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) activity assay

The first enzyme in *de novo* synthesis is γ -glutamylcysteine synthetase (GCS) that catalyzes glutamate and cysteine to form γ -glutamylcysteine. Then γ -glutamylcysteine and glycine forms GSH by second enzyme glutathione synthetase (GS). Activities of GCS and GS were determined by fluorescence-based microtiter plate assay originally developed by White *et al.* (White et al. 2003) with little modification. Briefly, 50 μ l of supernatant of homogenate was centrifuged in microcon-10 tubes for 20 min at 4 °C at $12,000 \times g$ to remove endogenous GSH and other inhibitors (Liu et al. 1998). Then the samples were washed with 0.1 M PBS and were centrifuged for 20 min to concentrate the protein. Final concentrates were tested for their protein concentration. For GCS activity assay, 50 μ l of sample was added to 50 μ l of GCS reaction cocktail containing 5 mM L-cysteine, 100 mM Tris, 10 mM ATP, 20 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, 2 mM serine, and 40 mM $MgCl_2$ at 37 °C. The GCS reaction was initiated by addition of 50 μ l of 2 mM cysteine. For GS activity assay, L-glutamic acid was substituted with 30 mM glycine, and L-cysteine was substituted with 3 mM γ -glutamylcysteine (γ -GC). After incubation for 20 min, the GCS and GS reaction were stopped by addition of 50 μ l of 200 mM 5-sulfosalicylic acid (SSA). Following deproteinization, the supernatants were incubated with 180 μ l of 10 mM 2, 3-Naphthalenedicarboxyaldehyde (NDA) to form NDA- γ -GC and NDA-GSH. Then NDA- γ -GC or NDA-GSH fluorescence intensity was measured (472 excitation/528 emission) on a fluorescence plate reader (Molecular Devices, Menlo Park, CA). The production of γ -GC and GSH was calculated with standard curves for NDA- γ -GC and NDA-GSH. Each assay was performed in duplicate. GCS and GS activity was expressed as nmol/min/mg protein.

2.7 Glutathione peroxidase (GPx) activity assay

Glutathione peroxidase (GPx) is a key enzyme in eliminating H_2O_2 and organic peroxides (Meister 1988). GPx activity was determined using a commercial kit supplied by Cayman Chemical. Briefly, 200 μ l of reaction mixture contained 20 μ l of supernatant of brain homogenate, 5.0 mM GSH, 0.1 mM NADPH, 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, and 0.1 unit of glutathione reductase (GR). The reaction was initiated by addition of 20 μ l of 0.2 mM cumene hydroperoxide at room temperature. The decrease in absorbance at 340 nm was recorded at 60 s intervals for 6 min. The rate of decrease in the absorbance is directly proportional to the GPx activity in the sample. Each assay was performed in duplicate and enzyme units were recorded as nmol NADPH oxidized/min/mg protein.

2.8 Glutathione reductase (GR) activity assay

During the detoxification, GSH is oxidized to GSSG. The lost GSH is regenerated by redox recycling, in which GSSG is reduced to GSH by glutathione reductase (GR) with a consumption of one NADPH. GR activity was assayed spectrophotometrically by monitoring the oxidation of NADPH to $NADP^+$ by GR at 340 nm. GR activities were assayed using Cayman GR assay kit. Briefly, 200 μ l of reaction mixture contained 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM GSSG, and 0.1 mM NADPH. The reaction was initiated by addition of 20 μ l of supernatant of brain homogenate. The decrease in absorbance at 340 nm was recorded at 60 s intervals for 6 min. Each assay was performed in duplicate and enzyme units were recorded as nmol NADPH oxidized/min/mg protein.

2.9 Glutathione-S-transferase (GST) activity assay

GSH participates the transportation of amino acids across cellular membranes by the γ -glutamyl cycle in which glutathione-S-transferase (GST) is required (Meister 1988). Three families of cytosolic soluble GSTs α (GSTA), μ (GSTM), π (GSTP) were known in the brain (Theodore et al. 1985; Board et al. 1990). Cayman's GST assay kit measured total GST activity (cytosolic and microsomal). Briefly, 200 μ l of reaction mixture contained 20 μ l of supernatant

of brain homogenate, 100 mM potassium phosphate (pH 6.5), 0.1% (v/v) Triton X-100, 5.0 mM GSH. The reaction was initiated by addition of 10 μ l of 20 mM 1-chloro-2, 4-dinitrobenzene (CDNB) at room temperature. The increase in absorbance at 340 nm was recorded at 60 s intervals for 6 min. The rate of increase in the absorbance is directly proportional to the GST activity in the sample. Each assay was performed in duplicate and enzyme units were recorded as nmol/min/mg protein.

2.10 γ -glutamyl transpeptidase (γ -GT) activity assay

γ -GT initiates the degradation of extracellular GSH by catalyzing the transfer of the γ -glutamyl moiety from GSH onto an acceptor molecule usually an amino acid to form γ -glutamyl-amino acid and cysteinylglycine, which then is broken down by dipeptidase to produce free cysteine for reuse. γ -GT activity was measured as described by Forman et al (Forman et al. 1995). Briefly, a 0.1 ml of supernatant of brain homogenates was added to 0.25 ml of a reaction mixture containing 50 mM Tris-HCl (pH 7.6), pH 8.6, 20 mM glycylglycine, 20 μ M γ -glutamyl-7-amino-4-methyl- coumarin (γ -glutamyl-AMC) and 0.1% (v/v) Triton X-100. The reaction was carried out at 37 °C for 30 min and terminated by adding 1.5 ml cold 50 mM glycine buffer. The fluorescence was measured at 440 nm with excitation wavelength of 370 nm. Product formation was calculated by compared with a standard curve of AMC. Each assay was performed in duplicate and enzyme units were recorded as nmol/min/mg protein.

2.11 Western blot for analyses of GCS protein

GCS heavy (GCS-HS) and light (GCS-LS) subunit protein was determined by Western blot as described by Liu et al (Liu 2002). The supernatants of homogenate were added with protease inhibitor cocktail then were processed as described for the analyses of GCS activity. For each sample, 30 μ g total protein was separated by 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE), and blots of the gels were incubated with the primary antibodies. Anti GCS-HS polyclonal antibody (Abcam Inc, Cambridge, MA) was diluted to 1:1000. Anti GCS-LS polyclonal antibody (Gift from University of Washington and Bristol-Myers Squibb Company, Seattle, Washington) was used at 1:2000. Films were exposed using ECL enhanced chemiluminescence (Amersham, Piscataway, NJ). The X-ray film was scanned and the density of band was quantified using Scion Image software (Scion Corporation, Frederick, MD). β -actin was used as sample loading control. After exposure to film, the membranes were incubated in 0.2M glycine (pH 2.5) at 50°C for 30 min and blotted again with mouse anti β -actin antibody (Abcam; 1:4000). The amount of protein was semi-quantified by scanning densitometry and was expressed as a relative value to the levels of β -actin blots.

2.12 Tyrosine hydroxylase (TH) immunohistochemistry and stereological cell counts

TH is widely used as an index of DA neurons (Fishell and van der Kooy 1989). The number of TH immunoreactive (TH-ir) cells was assessed using a stereological procedure described in previous studies (Ling et al. 2004a; Ling et al. 2004b; Ling et al. 2006). Briefly, following fixation, the brains were subsequently cut into 40 μ m coronal sections. Every sixth section was immunohistochemically processed for TH using mouse anti-rat TH antibody overnight (Immunostar, Stillwater MN; 1:20,000). The secondary antibody used was rat immunoglobulin against mouse IgG (rat absorbed) conjugated with biotin (Vector Laboratories, CA). Peroxidase conjugated avidin-biotin complex was applied following the secondary antibody. The chromogen solution contained 0.05% 3, 3'-diaminobenzidine (DAB), 0.5% nickel sulfate, and 0.003% H₂O₂ was used to obtain a black stain. The estimation of the total number of TH-ir neurons in the SN was determined using the computerized optical dissector method with StereoInvestigator software (MBF Bioscience, Williston, VT). The SN was anatomically identified as those cells lateral to the accessory optic track as previously described (Ling et al. 2004b). The total number (N) of TH-ir cells was calculated using the formula $N = NV \times VSN$,

where NV is the numerical density and VSN is the volume of the SN. TH-ir cells counts in the SN were done by an individual blinded to treatment history.

2.13 Statistical analyses

Data are expressed as mean $\pm$ SEM. The various measures were assessed using SPSS 11.5. Differences between LPS and saline-injected groups were analyzed by *t*-test. Two way or three-way analyses of variance (ANOVA) were used to analyze interaction of prenatal exposure, age and regions. Tukey's *post-hoc* analyses were used to detect statistical differences among the groups using $P < 0.05$ as significance values.

3. Results

3.1 Effects of acute LPS administration on glutathione and LPO

As summarized in table 1, unilateral supranigral LPS (10 μ g) injection in the rats resulted in a marked decrease in reduced GSH level ($32 \pm 6\%$) in the midbrain compared with saline-injected rats ($P < 0.01$). This loss was accompanied by an increase in oxidized glutathione (GSSG) ($39 \pm 13\%$; $P < 0.01$) level and a decline in GSH/GSSG ratio ($44 \pm 4\%$; $P < 0.01$). LPO is an index of lipid peroxidation. Analyses of LPO showed that 3 days following supranigral LPS injection there was a significant increase in LPO in the midbrain relative to group receiving saline injection ($P < 0.01$; Table 1).

3.2 Effects of acute LPS administration on enzyme activities of glutathione homeostasis

GCS catalyzes the first step reaction in *de novo* glutathione synthesis to form γ -glutamylcystein from cysteine and glutamate. GCS is composed of heavy and light subunits namely GCS-HS and GCS-LS. As summarized in Table 2, a single injection of LPS resulted in an increase ($57 \pm 13\%$) in GCS activity in midbrain compared with saline -injected animals ($P < 0.01$). Western blot assays showed that there were statistically significant increases ($28 \pm 8\%$; $P < 0.01$) of GCS-HS and GCS-LS protein levels ($21 \pm 10\%$; $P < 0.01$) in LPS-injected midbrain when compared with saline-injected animals (Fig. 1A and 1B). This indicated that GCS was up-regulated both in enzyme activity and protein expression in response to GSH loss following acute LPS injection.

GS catalyzes second step reaction in *de novo* GSH synthesis, in which γ -glutamylcysteine combines with glycine to generate GSH. GS activity was markedly increased in the midbrain of rats received acute LPS injection ($P < 0.001$; Table 2).

GSH is oxidized to GSSG during brain detoxification by GPx. GR is a reductase which brings oxidized glutathione (GSSG) back to GSH in redox reaction. Analyses of GPx and GR activities in LPS-injected animals showed that 3 days following LPS injection there was a marked increase (over two fold) in both GPx and GR activities in the midbrain ($P < 0.01$; Table 2). This indicated that GPx and GR were both up-regulated in enzyme activities in response to inflammation induced by LPS injection.

GST, and γ -GT are GSH-using enzymes, these enzyme activities were also measured in brain tissues. Acute LPS administration did not change γ -GT and GST activities ($P > 0.05$; Table 2).

3.3 Effects of prenatal LPS exposure on postnatal glutathione homeostasis

Overall, prenatal LPS exposure at embryonic day 10.5 led to GSH loss in cortex, striatum, midbrain, and cerebellum ($F_1, 64=27.44$, $P < 0.0001$) (Fig. 2A). Significant aging effects were also detected between 4 months and 17 months old animals ($F_1, 64=76.39$, $P < 0.0001$). There were no significant differences among these four regions ($F_3, 64=0.15$, $P=0.93$). Aging accelerated GSH loss in LPS animals as indicated by a positive interaction between prenatal

LPS exposure and aging ($F_{1, 64}=4.81$, $P=0.03$). In addition, prenatal LPS exposure resulted in a significant increase in GSSG content ($F_{1, 64}=30.62$, $P<0.0001$) and a decrease in GSH/GSSG ratio ($F_{1, 64}=49.68$, $P<0.0001$) compared with prenatal saline control (Fig. 2B and Fig. 2C). Similar to GSH, aging also produced age-related increase in GSSG level ($F_{1, 64}=44.67$, $P<0.0001$) and GSH/GSSG ratio ($F_{1, 64}=86.73$, $P<0.0001$). In addition, aging also accelerated prenatal LPS exposure-induced GSSG increase and GSH/GSSG ratio decrease ($F_{1, 64}=12.63$, $P<0.01$). There were no significant regional differences in GSSG levels ($F_{3, 64}=0.24$, $P=0.07$) and GSH/GSSG ratios ($F_{3, 64}=1.62$, $P=0.19$) among four brain regions examined.

3.4 Effect of prenatal LPS exposure on postnatal brain LPO

Both prenatal LPS exposure ($F_{1, 64}=17.93$, $P<0.0001$) and aging ($F_{1, 64}=117.43$, $P<0.0001$) caused LPO increases in the brain (Fig. 3). Unlike glutathione, there were significant differences among these four regions ($F_{3, 64}=4.28$, $P=0.01$) with a higher level in the midbrain ($P<0.05$), indicating more free radical, reactive oxygen species, or nitro species were generated in this area when animals when aging. Similarly, aging enhanced prenatal LPS exposure-induced LPO increase in 17 months old animals ($F_{1, 64}=6.12$, $P=0.02$).

3.5 Effects of prenatal LPS exposure on postnatal brain GCS and GS activities

Overall, prenatal LPS exposure led to a decrease in GCS activity in all regions examined ($F_{1, 64}=34.56$, $P<0.0001$; Fig. 4). However, there were no significant regional heterogeneities in GCS activity among these regions ($F_{3, 64}=0.98$, $P=0.40$). Furthermore, prenatal LPS exposure-caused GCS reduction became more pronounced in aged brains ($F_{1, 64}=4.09$, $P=0.01$). These changes were consistent with glutathione levels in these regions. GCS subunits protein analyses showed that only GCS-LS protein expression was significantly decreased in prenatally LPS-exposed brains when compared with saline control ($F_{1, 64}=14.69$, $P<0.0001$). Aging also acted as independent factor to cause GCS-LS protein decrease ($F_{1, 64}=16.27$, $P<0.0001$; Fig. 5B and 5D). A positive interaction was found between aging and prenatal LPS exposure on GCS-LS expression ($F_{1, 64}=10.40$, $P<0.0001$). In contrast, there were no significant changes in GCS-HS protein in terms of age and prenatal LPS exposure (Fig. 5A and 5C). There were no significant regional differences in GCS-HS and GCS-LS protein expressions among four regions examined.

Neither prenatal LPS exposure nor aging significantly altered GS activity (data not shown).

3.6 Effects of prenatal LPS exposure on postnatal brain GPx and GR activities

Overall, prenatal LPS exposure led to GPx activity increase in all regions examined when compared with prenatal saline controls ($F_{1, 64}=22.01$, $P<0.0001$; Fig. 6A). GPx activity was also increased when animals were aged ($F_{1, 64}=38.92$, $P<0.0001$). Similarly, GPx activity was higher in midbrain compared with other regions ($F_{3, 64}=3.67$, $P=0.01$). Furthermore, aging accelerated GPx activity increase in LPS animals ($F_{1, 64}=3.60$, $P=0.02$).

Analyses of GR activity in the brain showed that GR activity was significantly increased in prenatally LPS-exposed brains ($F_{1, 64}=5.75$, $P=0.02$; Fig. 6B). There was a significant increase in GR activity when animals were aged ($F_{1, 64}=58.64$, $P<0.0001$). In addition, a regional difference in GR activity among four brain regions was detected with highest activity in midbrain ($F_{3, 64}=6.14$, $P=0.001$). Aging did not enhance GR activity increase in LPS animals ($F_{1, 64}=0.29$, $P=0.59$).

3.7 Effects of prenatal LPS exposure on postnatal brain γ -GT and GST activities

As illustrated in figure 7A, both prenatal LPS exposure ($F_{1, 64}=11.30$, $P<0.0001$) and aging ($F_{1, 64}=57.83$, $P<0.0001$) produced an increase in γ -GT activity, suggesting that γ -glutamyl

cycle was up-regulated in aged or prenatally LPS-exposed animals. There was a statistical difference among these four regions ($F_3, 64=4.03, P=0.02$). However, aging did not accelerate γ -GT activity increase in LPS animals ($F_1, 64=0.93, P=0.34$).

There are three isoforms of GST in the brain. Total GSH enzymes activities analyses showed that prenatal LPS exposure led to GST activity increases in all regions examined ($F_1, 64=14.62, P<0.0001$; Fig. 7B). Similar to prenatal LPS, aging also caused GST activity increase ($F_1, 64=38.43, P<0.0001$). In addition, there was a significant regional difference in GST activity among four regions with higher activity in midbrain ($F_3, 64=12.12, P<0.0001$). However, GST activity was not higher in prenatally LPS-exposed brains compared with prenatal saline controls when animals were aged ($F_1, 64=2.79, P=0.10$).

3.8. Effects of prenatal LPS exposure on postnatal brain SN TH-ir cell counts

Overall, two-way ANOVA analysis on the TH-ir cell count showed that prenatal LPS exposure at embryonic day 10.5 produced a significant reduction in TH-ir cell counts in the SN of postnatal brains ($F_1, 16=51.55; P<0.0001$; Fig. 8). Significant aging effects were also detected between 4 months and 17 months old animals ($F_1, 16=14.05; P<0.0001$). Prenatal LPS exposure reduced TH-ir cell count by 24.7% at 4 months old relative to saline exposed controls at same age. This loss extended to 33.4% at 17 months old as a result of aging. The interaction between the prenatal treatments and aging was significant ($F_1, 16=6.46; P=0.02$), suggesting that aging accelerated DA neuron loss in the animals exposed prenatally to LPS.

4. Discussion

Studies from several laboratories indicated that both prenatal LPS exposure (Ling et al. 2002; Ling et al. 2004a; Ling et al. 2006) and supranigral LPS injection induced DA neuron loss (Castano et al. 1998; Herrera et al. 2000; Gao et al. 2002; Arimoto and Bing 2003). In case of prenatal LPS exposure, pups display many characteristics seen in patients with PD. These include DA neuron loss in nigra, DA loss in striatum, Lewy body-like inclusion formation, protein oxidation, and increase of proinflammatory cytokine expression along with increase of reactive microglia in brain (Ling et al. 2002; Ling et al. 2004a; Ling et al. 2006). One other striking feature of LPS animals is their hypersensitivity to secondary toxin exposure (Ling et al. 2004a; Ling et al. 2006). It is postulated that LPS animals may have reduced capacity to buffer oxidative products brought about by the secondary toxin. In case of supranigral LPS injection, the loss of DA neuron progresses over several weeks (Gao et al. 2002; Ling et al. 2006). In both cases, neuroinflammation is evident (Gao et al. 2002; Ling et al. 2006). The current study was designed to substantiate our hypothesis that glutathione metabolic disorder is a mechanism underlying increased susceptibility of LPS animals to postnatal toxin. Acute supranigral LPS injection to 4 months old animals were used for comparison to see if two paradigms produce similar consequence in brain glutathione homeostasis.

In current study, prenatal LPS exposure and acute supranigral LPS injection caused GSH loss along with increase in GSSG level and decrease in GSH/GSSG ratio. Although the outcomes were similar in two paradigms but the enzyme profiles were different between them. The GSH reduction in acute LPS injection model was associated with an increased GCS activity and higher levels of GCS-HS and -LS protein expressions. While prenatal LPS exposure-induced GSH reduction was associated with a decreased GCS activity and lower level of GCS-LS protein expression and unchanged GCS-HS protein level, suggesting a deficiency in *de novo* glutathione synthesis. In addition, since GCS expression was found decreased during aging (Liu 2002; Zhu et al. 2006), it was not surprising to see a synergy between prenatal LPS exposure and aging (Figure 4). In acute LPS injection model, increase in GCS activity was associated with an up-regulation of GCS-HS and -LS protein levels reflecting a normal

compensatory capability in younger animals. This finding was in agreement with a report by Kenchappa et al (Kenchappa and Ravindranath 2003).

Prenatal LPS exposure did not change GS activity but acute LPS injection did (significant increase in GS activity, Table 2). These data suggested that prenatal LPS exposure permanently down-regulates *de novo* glutathione synthesis.

Both prenatal LPS exposure and acute LPS injection produced increases in GPx and GR enzyme activities in GSH redox cycling. Study by Thiruchelvam et al. (Thiruchelvam et al. 2005) has suggested that over-expression of GPx and GR are protective. Thus, the increases in GPx and GR activities suggested a compensatory response to increases in oxidative stress which was indicated by the elevated LPO levels (Table 1 and figure 3) and protein oxidation (Ling et al. 2004a) in both prenatal LPS and acute LPS injection models.

GST and γ -GT both are GSH-using enzymes and participate in detoxification of exogenous and endogenous toxic substances (Meister 1988). GSH also participates the transportation of amino acids across cellular membranes while γ -GT initiates the degradation of extracellular GSH by catalyzing the transfer of the γ -glutamyl moiety from GSH onto an acceptor molecule (Griffith et al. 1978). Based on these, the increased GST and γ -GT activities in prenatal LPS model were best explained as a compensatory response to an increased oxidative stress. Lack of compensatory responses in acute LPS injection model may reflect an epiphenomenon due to acute tissue damage, such as edema and cell death, which conceal the elevated enzyme activities. Ahmad et al. (Ahmad et al. 2006) had reported that in 6-hydroxydopamine-lesioned animals glutathione dependent enzymes were reduced, which seems to support such view. In addition, tissue inflammation (Gao et al. 2002; Ling et al. 2004a) and TNF- α increase (Gao et al. 2002; Ling et al. 2004b; Ling et al. 2006) is apparent in both paradigms. The dispersion had ruled out a possibility that TNF- α directly mediates GST or γ -GT activity since TNF- α levels were increased in both models.

Regional differences were detected in GR, γ -GT, and GST activities in current study. GR was found increased in midbrain of LPS animals particularly in the midbrain of older animals. This is likely resulted from the fact that midbrain contains high level of DA which is not sequestered in synaptic vesicles, promoting a higher turnover of DA which produces hydroxyl radicals and hydrogen peroxide. In LPS animals, oxidative stress seems to be more prominent due to loss of DA neurons and proinflammatory state in the midbrain (Ling et al. 2004a). Aging may serve as an added factor in this regard as indicated by protein oxidation (Ling et al. 2004a) and increase in LPO levels (Zhu et al. 2006). Higher levels of hydroxyl radicals and hydrogen peroxide may deplete more GSH to cause a greater increase in GSSG, thus stimulating an increase in GR to remove excess GSSG. Similar mechanism may apply to the observed increase in GST activity. GST is a detoxification enzyme which catalyzes the glutathione moiety to a great variety of acceptor molecules including toxins, organic hydroperoxides, and lipid peroxides to form conjugates. These conjugates are then degraded in the γ -glutamyl cycle. Therefore, increase in oxidative stress in midbrain may stimulate an up-regulation of GST activity in the midbrain. γ -GT initiates the degradation of extracellular GSH by catalyzing the transfer of the γ -glutamyl moiety from GSH onto an acceptor molecule usually an amino acid to form γ -glutamyl-amino acid and cysteinylglycine, which then is broken down by dipeptidase to produce free cysteine for reuse. Because of its degradative function, increases in γ -GT activity would theoretically lead to a decrease in GSH level. In fact Sian et al. (Sian et al. 1994) have reported that the decrease in GSH in the substantia nigra in Parkinson's disease was due to an increase in γ -GT activity (Griffith et al. 1978), which was exactly what was observed in the prenatal LPS model. This data further validate prenatal LPS exposure as a model for Parkinson's disease.

In the cell count data, we consistently demonstrated that prenatal LPS effect on DA neuron loss. We also showed that aging effect was seen in both prenatally groups. The greater TH-ir cell loss in LPS animals at 17 months old was a result of both LPS exposure and aging. Thus, cell count data seems parallel with glutathione data where the glutathione homeostasis disturbance was most pronounced at 17 months of age. These suggest that the glutathione homeostasis disturbance could be causative and render DA neurons continue to loss when aging.

In conclusion, deficiency of glutathione in prenatally LPS-exposed animals may at least in part contribute to the increased susceptibility to secondary toxin exposure in a study reported previously (Ling et al. 2004a; Ling et al. 2006). These data also validate the scientific value and significance of prenatal LPS model in PD research for its characteristics resembling many pathological features seen in patients with PD. Current study also strongly supports multi-hit theory in PD pathogenesis (Cory-Slechta et al. 2005; Williams and Ramsden 2005).

Acknowledgements

This study was supported by NINDS NS045316, NIEHS 012307, USARMRA W81XWH-04-01-0365, and grant from the Michael J. Fox Foundation. The authors also thank Dr. Terrance J. Kavanagh at University of Washington for his generosity of providing GCS-LS antibody.

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Fig. 1. γ -Glutamylcysteine synthetase (GCS) subunit protein in the midbrain. Heavy subunit (GCS-HS) and light subunit (GCS-LS) of GCS were detected in the midbrain following supranigral saline (Sal) or lipopolysaccharide (LPS) injection. β -actin was used as a control of protein loading. (A) Representative Western blot images. (B) Relative optical density of immunoblot band. Data are the means $\pm$ SEM from 5 animals each group. ** $P < 0.01$, compared with saline control.

Fig.2.

Reduced glutathione (GSH), glutathione disulfide (GSSG) levels and GSH/GSSG ratio in the brain. Glutathione concentration was measured in cortex, striatum, midbrain and cerebellum in prenatally LPS or saline (Sal)-exposed rats at 4 and 17 months of age. (A): GSH; (B): GSSG; (C): Ratio of GSH/GSSG. Each bar from left to right in different regions represents Sal/4 months, LPS/4 months, Sal/17 months and LPS/17 months, respectively. Data are the means $\pm$ SEM from 5 animals each group. * $P < 0.05$, ** $P < 0.01$, compared with the corresponding group at 4 months of age. # $P < 0.05$, ## $P < 0.01$, compared with the corresponding saline group.

Fig. 3.

Lipid peroxide (LPO) level in the brain. LPO was measured in cortex, striatum, midbrain and cerebellum in prenatally LPS or saline (Sal)-exposed rats at 4 and 17 months of age. Each bar from left to right in different regions represents Sal/4 months, LPS/4 months, Sal/17 months and LPS/17 months, respectively. Data are the means $\pm$ SEM from 5 animals each group. * P < 0.05, ** P < 0.01, compared with the corresponding group at 4 months of age. ## P < 0.01, compared with the corresponding saline group.

Fig. 4.

γ -Glutamylcysteine synthetase (GCS) activity in the brain. GCS activity was measured in cortex, striatum, midbrain and cerebellum in prenatally LPS or saline (Sal)-exposed rats at 4 and 17 months of age. Each bar from left to right in different regions represents Sal/4 months, LPS/4 months, Sal/17 months and LPS/17 months, respectively. Data are the means $\pm$ SEM from 5 animals each group. $*P < 0.05$, $**P < 0.01$, compared with the corresponding group at 4 months of age. $^{\#}P < 0.05$, $^{##}P < 0.01$, compared with the corresponding saline group.

Fig. 5.

γ -Glutamylcysteine synthetase (GCS) subunit protein in the brain. Heavy subunit (GCS-HS) and light subunit (GCS-LS) of GCS were detected in cortex (COR), striatum (STR), midbrain (MID) and cerebellum (CER) in prenatally LPS or saline (Sal)-exposed rats at 4 and 17 months (m) of age. β -actin was used as a control of protein loading. (A, B) Representative Western blot images from four groups of animals; (C, D) Relative optical density of immunoblot band. Each bar from left to right in different regions represents Sal/4 months, LPS/4 months, Sal/17 months and LPS/17 months, respectively. Data are the means $\pm$ SEM from 5 animals each group. No significant changes of GCS-HS protein were observed in all groups. $**P < 0.01$, compared with the corresponding group at 4 months of age. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the corresponding saline group.

Fig.6.

Glutathione peroxidase (GPx) and glutathione reductase (GR) activities in the brain. (A) GPx and (B) GR activities were measured in cortex, striatum, midbrain and cerebellum in prenatally LPS or saline (Sal)-exposed rats at 4 and 17 months of age. Each bar from left to right in different regions represents Sal/4 months, LPS/4 months, Sal/17 months and LPS/17 months, respectively. Data are the means $\pm$ SEM from 5 animals each group. * $P < 0.05$, ** $P < 0.01$, compared with the corresponding group at 4 months of age. # $P < 0.05$, ## $P < 0.01$, compared with the corresponding saline group.

Fig. 7.

γ -Glutamyl transpeptidase (γ -GT) and glutathione S-transferase (GST) activities in the brain. (A) γ -GT and (B) GST activities were measured in cortex, striatum, midbrain and cerebellum in prenatally LPS or saline (Sal)-exposed rats at 4 and 17 months of age. Each bar from left to right in different regions represents Sal/4 months, LPS/4 months, Sal/17 months and LPS/17 months, respectively. Data are the means $\pm$ SEM from 5 animals each group. * P < 0.05, ** P < 0.01, compared with the corresponding group at 4 months of age. # P < 0.05, ## P < 0.01, compared with the corresponding saline group.

Fig. 8.

Upper panel: Representative photomicrographs of tyrosine hydroxylase immunoreactivity (TH-ir) in mesencephalon of the animals exposed prenatally to saline (Sal) or lipopolysaccharide (LPS) at 4 and 17 months old. A: Sal/4 months; B: LPS/4 months; C: Sal/17 months; D: LPS/17 months. Magnification bar = 0.25 mm. Lower panel: Figure shows TH-ir cell counts in the substantia nigra (SN). Data are the means $\pm$ SEM from 5 animals each group. * $P < 0.05$, compared with the corresponding group at 4 months of age. ## $P < 0.01$, compared with the respective saline group.

Table 1

Effects of supra-nigral LPS injection on GSH, GSSG and LPO levels in the midbrain

	GSH ($\mu\text{mol/g tissue}$)	GSSG ($\mu\text{mol/g tissue}$)	GSH/GSSG	LPO (nmol/g tissue)
Saline	1.14 ± 0.15	0.023 ± 0.002	50.5 ± 6.5	1.04 ± 0.05
LPS	$0.77 \pm 0.07^{**}$	$0.032 \pm 0.003^{**}$	$28.1 \pm 2.2^{**}$	$1.62 \pm 0.11^{**}$

Footnote: GSH: reduced form of glutathione. GSSG: oxidized form of glutathione. LPO: lipid peroxide. LPS: lipopolysaccharide. Values are expressed as mean $\pm$ SEM (n=5).

** P < 0.01, compared with the saline group.

Table 2

Effects of supra-nigral LPS injection on glutathione-related enzymes activities in the midbrain

	GCS	GS	GPx	GR	γ -GT	GST
Saline	1.01 $\pm$ 0.06	0.86 $\pm$ 0.05	6.74 $\pm$ 0.57	4.97 $\pm$ 0.64	0.69 $\pm$ 0.07	8.89 $\pm$ 0.89
LPS	1.59 $\pm$ 0.14**	1.32 $\pm$ 0.15**	14.02 $\pm$ 0.75**	10.49 $\pm$ 1.42**	0.79 $\pm$ 0.09	7.98 $\pm$ 0.67

Footnote: All the enzyme activities are expressed as nmol/min/mg protein. GCS: γ -glutamylcysteine synthetase. GS: glutathione synthetase. GR: glutathione reductase. GPx: glutathione peroxidase. γ -GT: γ -glutamyl transpeptidase. GST: glutathione S-transferase. Values are expressed as mean $\pm$ SEM (n=5).

**

P < 0.01, compared with the saline group.