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Research Report

Down-regulation of glutaredoxin by estrogen receptor antagonist renders female mice susceptible to excitatory amino acid mediated complex I inhibition in CNS

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L-BOAA, β-*N*-oxalyl-amino-L-alanine
GSH, glutathione
GSSG, oxidized glutathione
PrSSG, Protein–glutathione mixed disulfides

ABSTRACT

β-*N*-oxalyl-amino-L-alanine, (L-BOAA), an excitatory amino acid, acts as an agonist of the AMPA subtype of glutamate receptors. It inhibits mitochondrial complex I in motor cortex and lumbosacral cord of male mice through oxidation of critical thiol groups, and glutaredoxin, a thiol disulfide oxido-reductase, helps maintain integrity of complex I. Since incidence of neurolathyrism is less common in women, we examined the mechanisms underlying the gender-related effects. Inhibition of complex I activity by L-BOAA was seen in male but not female mice. Pretreatment of female mice with estrogen receptor antagonist ICI 182,780 or tamoxifen sensitizes them to L-BOAA toxicity, indicating that the neuroprotection is mediated by estrogen receptors. L-BOAA triggers glutathione (GSH) loss in male mice but not in female mice, and only a small but significant increase in oxidized glutathione (GSSG) was seen in females. As a consequence, up-regulation of γ-glutamyl cysteinyl synthase (the rate-limiting enzyme in glutathione synthesis) was seen only in male mouse CNS but not in females. Both glutathione reductase and glutaredoxin that reduce oxidized glutathione and protein glutathione mixed disulfides, respectively, were constitutively expressed at higher levels in females. Furthermore, glutaredoxin activity in female mice was down-regulated by estrogen antagonist indicating its regulation by estrogen receptor. The higher constitutive expression of glutathione reductase and glutaredoxin could potentially confer neuroprotection to female mice.

1. Introduction

Neurolathyrism is a type of motor neuron disease involving the upper motor neurons, degeneration of anterior horn cells and loss of axons in the pyramidal tracts in the lumbar spinal cord in humans (Roy, 1988). Neurolathyrism is caused by consumption of the drought resistant chickling pea from plant *Lathyrus sativus* that contains the glutamate agonist β-*N*-oxalyl-amino-L-alanine (L-BOAA, also known as β-*N*-oxalyl-α-β-diamino propionic acid, β-ODAP). L-BOAA mediates neurotoxicity through the AMPA subtype of glutamate receptor (Sriram et al., 1997).

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L-BOAA causes glutathione (GSH) loss and increase in protein–glutathione mixed disulfides (PrSSG) that results in inhibition of mitochondrial complex I, a major component of the mitochondrial electron transport chain, in motor cortex (MC) and lumbosacral cord (LSC) of male mice. The inhibition of mitochondrial complex I activity by L-BOAA presumably occurs through glutathionylation of critical thiol groups in subunits of complex I since it can be reversed in vitro by thiol reductants (Kenchappa et al., 2002; Taylor et al., 2003). Glutaredoxin (also known as thioltransferase; Grx1; EC 1.8.4.2), a thiol-disulfide oxido-reductase, specifically and efficiently reduces glutathionylated proteins to protein thiols (Chrestensen et al., 2000; Gravina and Mieyal, 1993). It is essential for maintenance of complex I function in normal conditions (Kenchappa and Ravindranath, 2003b) and its up-regulation is critical for recovery of complex I function following L-BOAA administration (Kenchappa et al., 2002).

In humans, the incidence of neurolathyrism is more common in men (1.5 times more), while women are less prone to the disease although consumption of the chickling pea is not significantly different (Roy, 1988). This gender difference in the neurotoxic response to L-BOAA is reflected in the mouse model of L-BOAA toxicity (Sriram et al., 1998). The animal model of L-BOAA toxicity not only mimics the gender difference but also the site specificity of L-BOAA action wherein only motor cortex and lumbosacral cord are affected (Sriram et al., 1998). We, therefore, examined the molecular mechanisms underlying the neuroprotection against excitotoxicity seen in female mice using this model.

2. Results

2.1. *Complex I activity is unaltered in female mouse brain after L-BOAA administration*

Male and female mice were administrated a single dose of L-BOAA (10 mg/kg body weight, s.c.). Complex I activity was significantly decreased in motor cortex and lumbosacral cord of male mice at 0.5 h after L-BOAA administration (39% lower than controls, Fig. 1a). In female mice, there was no significant change in complex I activity in motor cortex and lumbosacral cord as compared to respective controls after L-BOAA administration (Fig. 1a). Mitochondria obtained from lumbosacral cord of male mice treated with L-BOAA were incubated with DTT at 37 °C. DTT treatment reversed complex I inhibition caused by L-BOAA in lumbosacral cord, indicating that the inhibition is caused by oxidation of critical thiols of complex I subunits (Fig. 1b).

2.2. *Estrogen receptor antagonist renders female mice vulnerable to L-BOAA mediated complex I inhibition*

When sagittal slices of male mouse brain were preincubated with 17β-estradiol (100 μM) for 0.5 h prior to exposure to L-BOAA, they were protected from L-BOAA mediated complex I loss (Fig. 2a). Female mouse brain slices pretreated with tamoxifen (an estrogen receptor modulator; Shiau et al., 1998) were vulnerable to L-BOAA (Fig. 2b). Pretreatment with

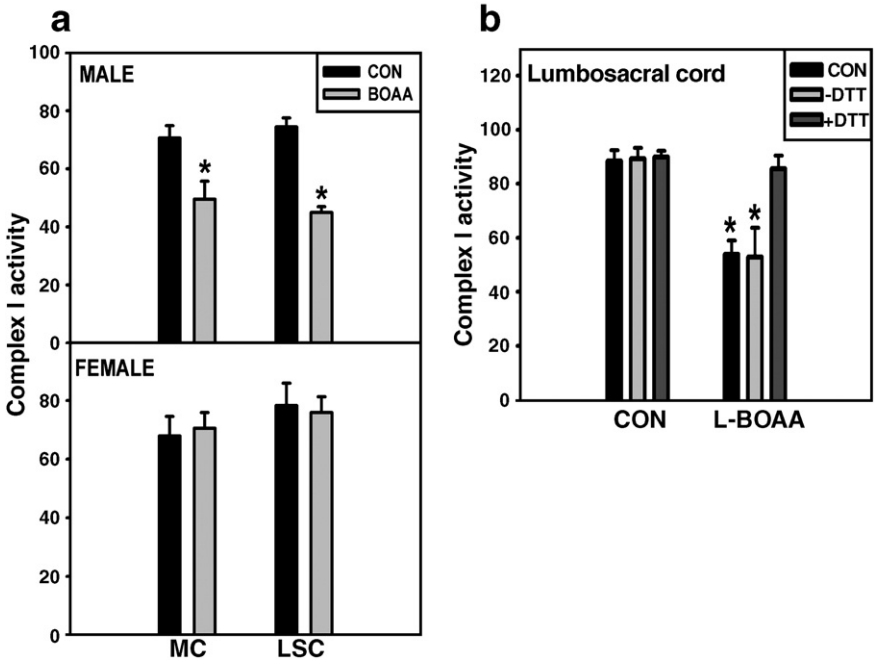

Fig. 1 – Effect of L-BOAA on complex I activity in male and female mouse brain regions. (a) Male and female mice were administered a single dose of L-BOAA (10 mg/kg body weight, s.c.), and animals were killed 0.5 h later (gray bars). Control animals received vehicle alone (black bars). Complex I activity was estimated in motor cortex and lumbosacral cord of male and female mice. The activity is expressed as nanomoles of NADH oxidised/min/mg protein. Values are mean ± SEM (n=4 animals). Asterisks indicate values significantly different from vehicle treated controls (P<0.001). (b) Mitochondria were isolated from lumbosacral cord of male mice treated with L-BOAA (10 mg/kg body weight s.c.) for 1 h. Mitochondrial samples were incubated with (+DTT) and without (–DTT) for 30 min at 37 °C, and complex I activity was measured.

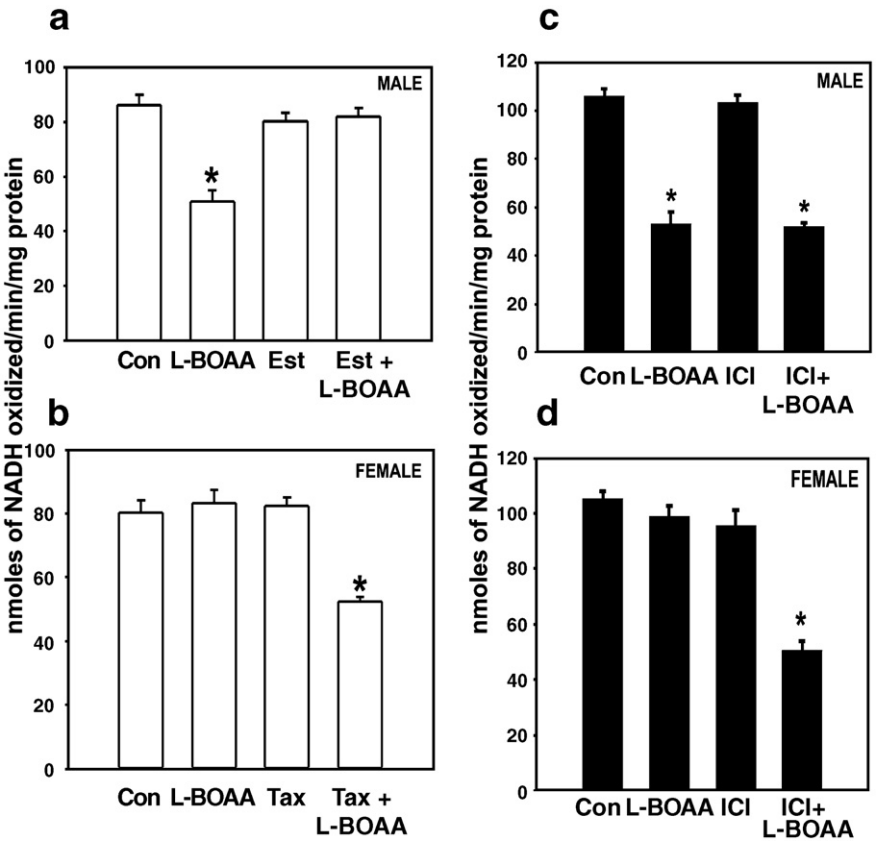

Fig. 2 – Effect of 17β-estradiol, tamoxifen and ICI 182,780 pretreatment on L-BOAA toxicity in male and female mouse brain slices. Sagittal slices prepared from male mouse brain were preincubated with estrogen (100 μM) in ACSF for 0.5 h followed by incubation with L-BOAA (1 nM) for 1 h. Complex I activity was measured in mitochondrial preparation from brain slices. Values are mean±SEM (n=5 slices). Asterisks indicate values significantly different from respective controls (P<0.001). Sagittal slices prepared from female mouse brain were preincubated with tamoxifen (10 nM) in ACSF for 0.5 h followed by incubation with L-BOAA (1 nM) for 1 h. Sagittal slices from male and female mouse were preincubated with ICI 182,780 (1 nM) for 0.5 h followed by incubation with L-BOAA (1 nM) for 1 h. Complex I activity was measured in mitochondrial preparation from above brain slices. Values are mean±SEM (n=5 slices). Complex I activity was expressed as nanomoles of NADH oxidized/min/mg protein. Asterisks indicate values significantly different from respective controls (P<0.001).

ICI 182,780, an estrogen antagonist (1 nM) for 0.5 h followed by incubation with L-BOAA (1 nM) for 1 h, abolished the neuroprotection seen in females and resulted in significant inhibition of complex I activity (Figs. 2c and d) in female, similar to that seen in males.

2.3. *GSH levels and γ-GCS expression are unaltered in female mouse brain after L-BOAA administration*

Total GSH content was measured 1 h after L-BOAA administration in motor cortex and lumbosacral cord regions of the CNS affected by L-BOAA. There was a significant decrease in GSH levels in the motor cortex and lumbosacral cord of the male mice (31% and 35% lower than controls, respectively, Fig. 3a). We estimated the levels of mRNA and activity of γ-glutamyl cysteine synthetase (the rate-limiting enzyme in GSH biosynthesis, γ-GCS) in male and female CNS regions after L-BOAA treatment. One hour after a single dose of L-BOAA, a significant increase in the levels of both mRNA and activity of γ-GCS was noted in motor cortex and lumbosacral cord of male mice, while they were similar to controls in females (Figs. 3b–d).

2.4. *Higher expression of glutathione reductase and glutaredoxin protects female mice against L-BOAA toxicity*

GSSG levels in the motor cortex of female mice increased 1 h after L-BOAA treatment (240% higher than corresponding control, Fig. 4a) indicating the generation of oxidative stress but this did not further translate into loss of GSH. Glutathione reductase (the key enzyme involved in reduction of GSSG to GSH) activity was higher in female mouse brain compared to male (Fig. 4b). When we examined glutaredoxin activity in CNS regions from female mice and compared it with male mice, we found that the activity in lumbosacral cord and motor cortex of female mice was significantly higher (197% and 196% respectively, Fig. 4c). In order to determine if glutaredoxin expression was regulated by estrogen through estrogen receptor(s) mediated signaling, we treated animals with ICI 182,780 (antagonist of both α and β estrogen receptor) and

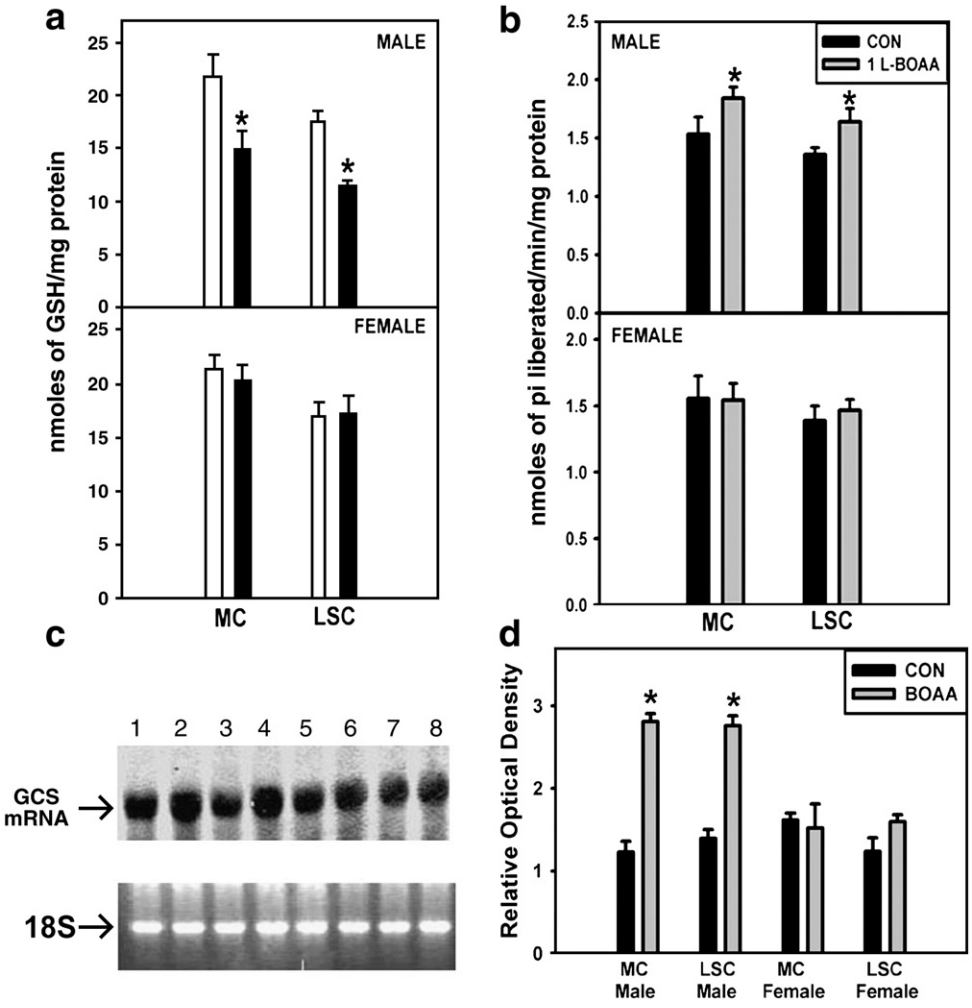

Fig. 3 – Change in (a) GSH levels, (b) γ-GCS activity and (c) mRNA levels of γ-GCS heavy chain subunit in male and female mice following L-BOAA exposure. Male and female mice were administered a single dose of L-BOAA (10 mg/kg bodyweight, s.c.), and animals were killed 1 h later (black bars). Control animals received vehicle alone (white bars). (a) GSH level was estimated in motor cortex (MC) and lumbosacral cord (LSC) of male and female mice. Values are mean ± SEM (n=4 animals) (b). γ-GCS activity was measured in motor cortex (MC) and lumbosacral cord (LSC) of male and female mice from control (black bars) and L-BOAA treated animals (gray bars). Values are mean ± SEM (n=4 animals). Asterisks indicate values significantly different from vehicle treated controls (P<0.001) (b). (c) A representative northern blot and corresponding 18S rRNA band of the total RNA from motor cortex (MC; lanes 1 and 2 for males, 5 and 6 for females) from controls (lanes 1 and 5) and L-BOAA treated (lanes 2 and 6) animals, respectively. Northern blot of RNA from lumbosacral cord (LSC; lanes 3 and 4 for males; 7 and 8 for females) from controls (lanes 3 and 7) and L-BOAA treated (lanes 4 and 8) animals is also depicted. (d) Densitometric analyses of northern blot representing relative intensity of the hybridized bands from controls (black bars) and L-BOAA treated animals (gray bars). Values are mean ± SEM (n=3 individual experiments). Asterisks indicate values significantly different from controls (P<0.001).

measured glutaredoxin activity in female mice CNS. Blockade of estrogen receptor(s) led to significant decrease in glutaredoxin activity (26% and 16% respectively) in lumbosacral cord and motor cortex (Fig. 4d).

2.5. *Immunohistochemical localization of estrogen receptor α and β in mouse brain*

Immunohistochemical studies demonstrated the presence of both α and β estrogen receptors in the cortex and lumbar spinal cord of mouse brain (Fig. 5) indicating that estrogen receptor mediated signaling could potentially occur at these sites.

3. Discussion

Estrogen, a neuroprotective agent (Behl, 2002; Dubal et al., 1998; Green and Simpkins, 2000; Hurn and Macrae, 2000), acts by two pathways at pharmacological (non-classical) and physiological (classical) concentrations. At pharmacological concentration, estrogen has high intrinsic antioxidant activity

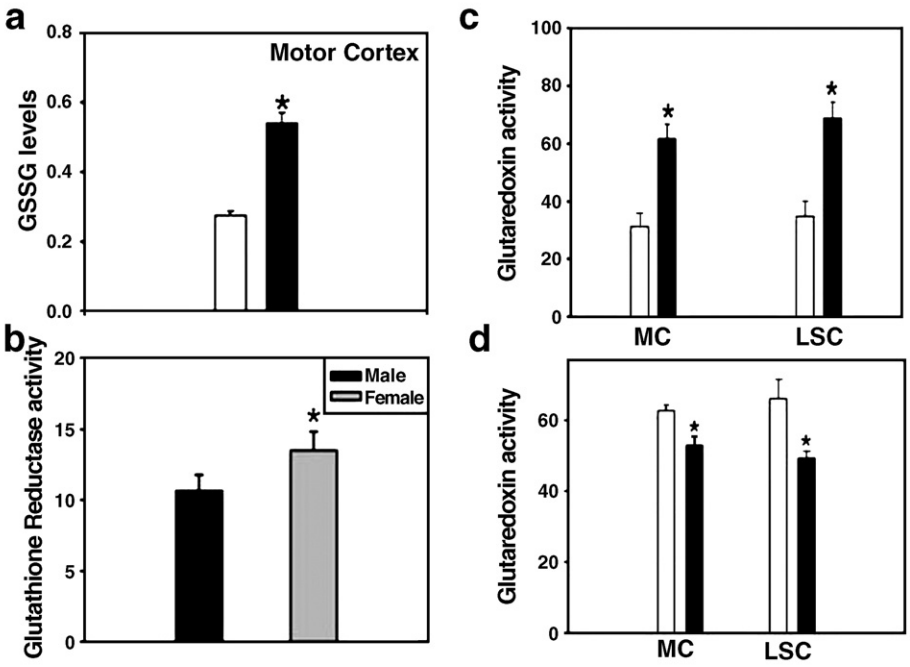

Fig. 4 – Effect of L-BOAA on GSSG levels and glutathione reductase and glutaredoxin activities: (a) Male and female mice were administered a single dose of L-BOAA (10 mg/kg bodyweight, s.c.), and animals were killed 1 h later. Control animals received vehicle alone. GSSG levels were estimated in motor cortex of female mice and are expressed as nanomoles of GSSG/mg protein. (b) Glutathione reductase activity was measured in male and female mouse brain and expressed as nanomoles of NADPH/min/mg protein. (c) Glutaredoxin activity was measured in motor cortex (MC) and lumbosacral cord (LSC) of male (white bars) and female mice (black bars) (c). (d) Female mice were treated with vehicle (white bars) or ICI 182,780 (1 mg/kg body weight) daily for 15 days (black bars), and glutaredoxin activity was measured. Values are mean ± SEM (n = 3 animals) expressed as nanomoles of NADPH utilized/min/mg protein. Asterisks indicate values significantly different from males (as in 'b') or controls (as in 'd'; P < 0.01).

(Liu et al., 1992; Sugioka et al., 1987) and 17β-estradiol at pharmacological concentration up to 1.0 μM inhibits lipid peroxidation and protects neurons against oxidative stress and glutamate-induced excitotoxicity, in vitro (Behl, 2002). In vivo, the physiological mode of estrogen action is mediated by estrogen receptors and involves induction or repression of gene expression leading to long-term genetic and physiological response. The precise molecular and cellular mechanisms underlying the physiological mode of estrogen mediated neuroprotection remain unclear. In this study, we demonstrate that estrogen regulates the expression of two key enzymes involved in maintenance of redox status, namely, glutaredoxin and glutathione reductase, and thereby helps maintain the redox status of proteins including mitochondrial complex I although there are no significant differences in the levels of GSH or the rate-limiting enzyme for GSH synthesis, γ-glutamyl cysteine synthase.

Earlier studies from our laboratory have shown that female mice are resistant to L-BOAA toxicity (Sriram et al., 1998). The early, reversible events (Kenchappa et al., 2002) in motor cortex and lumbosacral cord following L-BOAA administration involve loss of GSH, up-regulation of the glutaredoxin and γ-glutamyl cysteinyl synthase mRNA and protein (Kenchappa et al., 2002; Kenchappa and Ravindranath, 2003a). None of these responses was observed in female mice (Figs. 1 and 3).

During oxidative stress, GSH is oxidized to GSSG (oxidized glutathione), which can modify thiol groups in proteins to protein–glutathione mixed disulfides (PrSSG) at available cysteine residues if not extruded from the cell or reduced by glutathione reductase (Ravindranath and Reed, 1990). In brain, extrusion of GSSG during oxidative stress is not observed, however, extensive oxidative modification of protein thiols is seen as indicated by dramatic increase in PrSSG levels (Kenchappa et al., 2002; Shivakumar and Ravindranath, 1992).

In the present study, total GSH levels were unaffected in female mouse brain regions following L-BOAA treatment, while there was significant loss of GSH (30% decrease) in males. Oxidized GSH (GSSG) normally represents about 1% of total GSH in cells. A small but significant increase in GSSG level (2.4-fold increase over control representing 2.4% of total GSH) was seen in female mouse brain indicating that L-BOAA exposure generated oxidative stress in females, but this did not translate into loss of GSH as seen in male mouse brain. The lack of significant loss of GSH in females indicates that protein thiol modification, if any, occurring through formation of PrSSG is not sustained and is reduced back to protein thiol (PrSH) and GSH, thus maintaining GSH levels.

Glutathionylation of proteins can lead to their inactivation. Complex I contains several cysteine residues in its active site (Dupuis et al., 1991), and facile glutathionylation of thiol groups in the 51 and 75 kDa subunits of complex I by GSSG

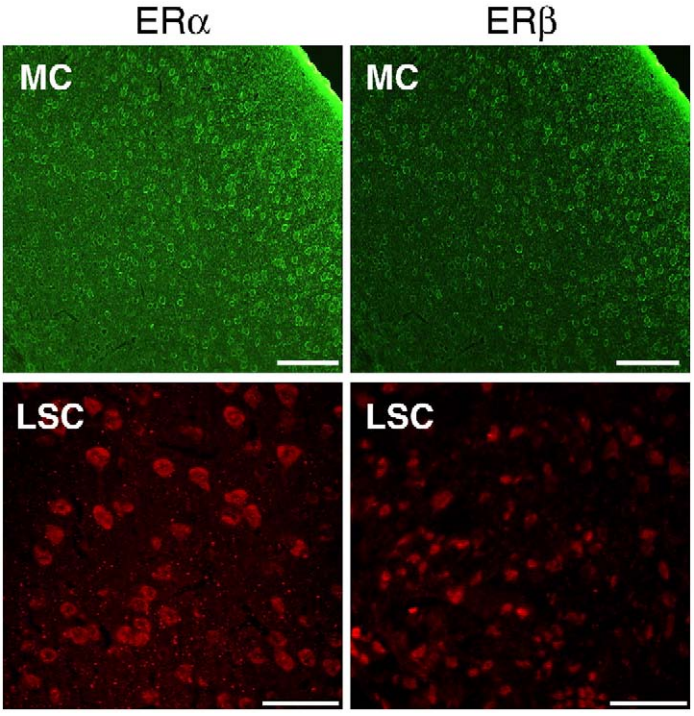

Fig. 5 – Immunohistochemical localization of estrogen receptor α and β in female mouse brain: Estrogen receptors α and β are expressed in cortical neurons (scale bar=50 μm) and in the lumbar spinal cord (scale bar=25 μm).

results in inhibition of the enzyme activity (Taylor et al., 2003) and increase in superoxide production (Taylor et al., 2003). Glutaredoxin efficiently and specifically catalyzes the reactivation of proteins oxidatively inactivated by S-glutathionylation (Chrestensen et al., 2000) and is critical for the maintenance and recovery of complex I activity under normal conditions and following oxidative stress (Kenchappa and Ravindranath, 2003b; Kenchappa et al., 2002). The facile reduction of PrSSG by glutaredoxin in the CNS of female mice may help to maintain thiol homeostasis and prevent mitochondrial dysfunction. This was also substantiated by higher glutathione reductase activity seen in female mice compared to male. The higher activity of glutathione reductase in female mice could potentially recycle the GSSG formed due to oxidative stress caused by L-BOAA back to GSH.

Glutaredoxin is expressed constitutively in higher levels in motor cortex and lumbosacral cord of female mice compared to males. Such gender difference is not noted in the expression of γ-GCS, the rate-limiting enzyme in GSH synthesis (Figs. 3a, b and c) or in the levels of GSH. Furthermore, glutaredoxin activity is decreased by the estrogen antagonist ICI 182,780, indicating that glutaredoxin is regulated through estrogen receptors. In male mice, ICI 182,780 did not have any effect (Fig. 2) indicating the inherent differences in response to estrogen receptor antagonist between male and female animals. Estrogen receptors α and β are localized in cortical neurons and in spinal cord (Fig. 5). In addition, estrogen receptors are localized primarily in neurons of anterior olfactory nucleus, cerebral cortex, red nucleus, locus ceruleus, hippocampus and motor trigeminal nucleus (Shughrue et al., 1997). Glutaredoxin is also localized in similar cell population (Balijepalli et al., 1999; Kenchappa et al., 2002). However, it is to be determined which of the estrogen receptors α or β is involved in the regulation of glutaredoxin.

Estrogen is known to regulate the expression of several neuroprotective genes. These include neurotrophic factors such as nerve growth factor, brain derived neurotrophic factor (BDNF), neurotrophin 3, their receptors TrkA–C and p75 and insulin-like growth factor 1 (Blurton-Jones et al., 1999; Sohrabji et al., 1995). The promoter region of anti-apoptotic genes Bcl2 and $BclX_L$ contains ERE, and estrogen enhances their transcription in vitro (Pike, 1999). A third group of genes regulated by estrogen in neurons is structural proteins such as neurofilament proteins, microtubulin-associated proteins, TAU and GAP43 (Brueggemeier et al., 2001; Ferreira and Caceres, 1991; Scoville et al., 1997; Shughrue and Dorsa, 1993; Reyna-Neyra et al., 2002). In addition to the above, glutaredoxin, the thiol-disulfide oxido-reductase, and glutathione reductase (Fig. 6) may represent a fourth class of genes that are regulated by estrogen and are involved in neuroprotection particularly those related to mitochondrial function.

4. Experimental procedures

L-BOAA was obtained from Research Biochemicals Inc., USA. Cysteinyl-glutathione disulfide was purchased from Toronto Research Chemicals (Toronto, Canada). TRI reagent was purchased from Molecular Research Inc (Cincinnati, OH, USA). A 390 bp fragment of the cDNA to γ-glutamyl cysteine synthetase (Genbank accession numberJ05181) representing 82–471 bp was a gift from Dr. T. Kavanagh (University of Washington, USA). Ubiquinone 1 was obtained as a gift from Eisai Pharmaceutical Company (Tokyo, Japan). ICI 182,780 was

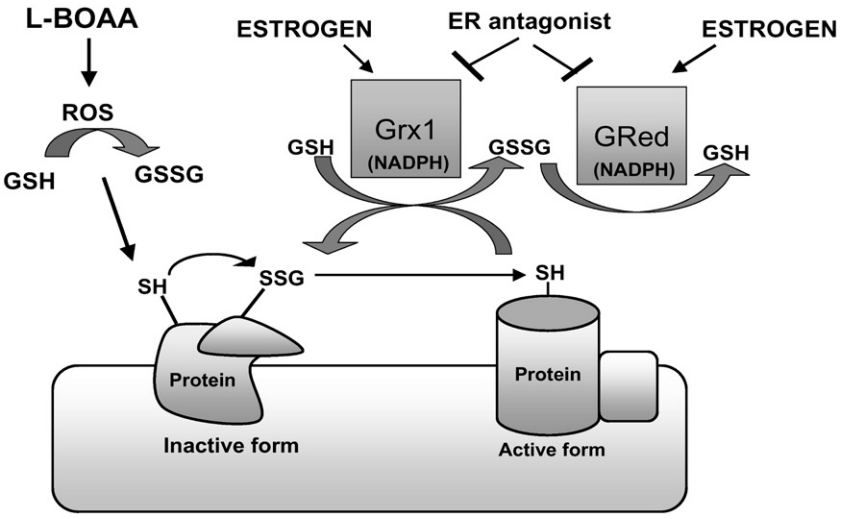

Fig. 6 – Glutathione and protein thiol homeostasis following L-BOAA treatment. L-BOAA triggers loss of glutathione (GSH) in male mice due to production of reactive oxygen species (ROS) that results in glutathionylation of proteins leading to formation of protein–glutathione mixed disulfides (PrSSG). This results in inhibition of enzymes, which have thiol groups in their active site, such as complex I. In female mice, L-BOAA administration does not lead to loss of GSH but only a small increase in GSSG (oxidized glutathione). Higher constitutive expression of glutaredoxin (Grx1) and glutathione reductase (GRed) may help to protect female mice from excitotoxins, such as L-BOAA by reducing PrSSG to PrSH (protein thiols) and GSSG to GSH, respectively, thereby preventing protein modification through glutathionylation.

obtained from Tocris Cookson (Avonmouth, UK). Northern blot analysis was performed using digoxigenin-labeling kit from Roche Biochemicals (Mannheim, Germany). Reductacryl reagent (dithiothreitol immobilized on polyacrylamide resin) was purchased from Calbiochem, Dramstadlt, Germany. Antibodies for estrogen receptors α and β were obtained from Santa Cruz Biotechnology Inc, California, USA. All other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Company (St. Louis, MO, USA) or Qualigens (Mumbai, India).

4.1. *Animals*

Male and female Swiss albino mice (3–4 months old, 25–30 g) were obtained from the Central Animal Research Facility of the National Institute of Mental Health and Neurosciences. Animals had free access to pelleted diet (Lipton Calcutta, India) and water *ad libitum*. All animal experiments were carried out according to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering, to reduce number of animals used and to employ alternatives to in vivo techniques, if available.

In vitro studies were performed using sagittal slices (representing most brain regions) of female mouse brain of 1 mm thickness, as described earlier (Pai et al., 1991). The slices were incubated at 37 °C in artificial cerebrospinal fluid (ACSF), pH 7.4, containing sodium chloride (122 mM), potassium chloride (3.1 mM), calcium chloride (1.3 mM), magnesium sulfate (1.2 mM), glucose (10 mM) and glycyl glycine (30 mM) in an oxygen rich atmosphere with or without L-BOAA (1 nM) for 1 h. In some experiments slices were pretreated with ICI 182,780 (1 nM) for 0.5 h prior to incubation with L-BOAA (1 nM) for 1 h. Following incubation, the slices were removed and homogenized as such in 0.25 M sucrose, and centrifuged at 1000×*g* for 10 min. The post-nuclear supernatant was recentrifuged at 14,000×*g* for 30 min to obtain mitochondrial pellet. Mitochondrial pellet was suspended in sucrose (0.25 M) and freeze–thawed prior to measurement of complex I activity.

For in vivo studies, L-BOAA (10 mg/kg body weight, s.c.) was dissolved in normal saline and animals were killed at 0.5 or 1 h after L-BOAA treatment. Motor cortex and lumbosacral segment of the spinal cord were dissected out and processed for experimental procedures.

4.2. *Processing of tissue*

CNS regions were dissected out and immediately processed for measurement of activities of complex I, glutaredoxin and γ-glutamyl cysteine synthetase (γ-GCS) and estimation of total GSH levels. Tissue was homogenized in 0.25 M sucrose and centrifuged at 1000×*g* for 10 min to obtain the post-nuclear supernatant. An aliquot of the post-nuclear supernatant was used for estimation of γ-GCS activity. The remaining portion of post-nuclear supernatant was centrifuged again at 14,000×*g* for 30 min to obtain the mitochondrial pellet. The post-mitochondrial supernatant was used for estimation of glutaredoxin activity. The mitochondrial pellet was suspended in sucrose (0.25 M) and freeze–thawed prior to assay of complex I activity. For the assay of GSH, the tissue was frozen immediately in liquid nitrogen and homogenized in 9 volumes of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. An aliquot of homogenate was added to an equal volume of 5-sulfosalicylic acid (1%, w/v), mixed and centrifuged at 10,000×*g* for 10 min, and the supernatant was used for estimation of GSSG and total GSH. The protein

concentration was estimated by a dye-binding method as described earlier (Balijepalli et al., 1999).

4.3. Estimation of glutathione (GSH+GSSG)

Total glutathione (GSH+GSSG) was estimated by the enzymatic recycling method (Sriram et al., 1998). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4) containing 0.8 mM dithiobisnitrobenzoic acid and 0.5 U/ml glutathione reductase in a total volume of 0.44 ml. The acid-soluble tissue supernatant (0.01 ml) prepared as described above was added. The reaction was initiated by adding 0.05 ml of 1.2 mM NADPH. The increase in absorbance was measured at 412 nm over 3 min. A standard curve was generated with known amount of GSSG. GSSG was estimated using the acid-soluble supernatant described above. An aliquot of the acid-soluble supernatant (0.1 ml) was also transferred to a test tube containing solid sodium bicarbonate (50 mg). Immediately, 10 μl of iodoacetic acid (0.2 M) was added and the tube was kept in dark at room temperature for 1 h. The contents of the tube were centrifuged briefly, and an aliquot was used for GSSG estimation (Sriram et al., 1997).

4.4. Assay of NADH: ubiquinone oxido-reductase (complex I) activity

Complex I was assayed in mitochondrial preparations as rotenone-sensitive NADH-ubiquinone oxido-reductase (Kenchappa et al., 2002; Sriram et al., 1998). The assay was performed in 35 mM potassium phosphate buffer (pH 7.4) containing sodium cyanide (2.65 mM), magnesium chloride (5 mM), EDTA (1 mM), bovine serum albumin (1 mg/ml) and antimycin (2 μg/ml). Brain mitochondria (70–80 μg) and ubiquinone 1 (0.05 mM) were added to the assay buffer to a final volume of 0.48 ml. After preincubation of the reaction mixture at room temperature for 2 min, the reaction was initiated by addition 0.02 ml of 5 mM NADH solution. Test and blank reactions were run separately. The rate of decrease of absorbance at 340 nm was monitored over 3 min. The assay was also carried out in the presence of rotenone to determine rotenone-sensitive enzyme activity. Less than 2% of the total activity was rotenone insensitive. The enzyme activity is expressed as nanomoles of NADH oxidized/min/mg protein.

4.5. Assay of glutaredoxin activity

Glutaredoxin activity was estimated in post-mitochondrial supernatant using cysteinyl-glutathione disulfide as substrate as described elsewhere (Balijepalli et al., 1999). Post-mitochondrial supernatant from brain region or spinal cord was suspended in 0.11 M potassium phosphate buffer (pH 7.4) containing 0.5 mM GSH, 2.4 U/ml glutathione reductase and 3.5 mM NADPH. The reaction was initiated by addition of the substrate, cysteinyl-glutathione disulfide (100 mM). The decrease in absorbance of NADPH at 340 nm was measured for 3 min. Blanks were run simultaneously without addition of post-mitochondrial supernatant. The net enzymatic rate was obtained by subtraction of non-enzymatic rate from total rate. The enzyme activity was expressed as nanomoles of NADPH oxidized/min/mg protein.

4.6. Assay of γ-glutamyl cysteine synthetase (γ-GCS) activity

γ-GCS activity was measured in post-nuclear supernatant as described by Chung et al. (1982) with minor modifications. The reaction mixture consisted of ATP (50 mM), glutamate (100 mM), bovine serum albumin (50 μg) and post-nuclear supernatant (70–100 μg of protein). The volume was made up to 125 μl with 100 mM Tris–HCl, (pH 8.2) and the reaction was initiated by addition of the substrate, L-α-amino butyrate (100 mM). The reaction mixture was incubated at 37 °C for 30 min. After incubation, reaction was stopped using 125 μl of trichloroacetic acid (10% w/v). This was followed by addition of 1.0 ml of ammonium molybdate (1.6% w/v) and 0.4 ml of ferrous sulfate (1% w/v) solution. Finally, the reaction mixture was centrifuged at 5000×*g* for 5 min and the absorbance was measured at 660 nm. Blank reactions without addition of substrate were run simultaneously for each sample. A standard curve was set up with known concentrations of potassium dihydrogen phosphate (10–200 nmol). The amount of phosphate (Pi) released by the enzyme was calculated from the standard curve, and γ-GCS activity was expressed as μmol of Pi released/h/mg protein.

4.7. Assay of glutathione reductase activity

The enzyme reduces glutathione disulfide to reduced glutathione with concomitant oxidation of NADPH to $NADP^+$. The formation of $NADP^+$ was measured by following a change in absorbance at 340 nm. Reaction mixture contained GSSG (1.6 mM) and protein (20–70 μg). The volume was made up to 1.0 ml by the addition of NADPH (0.05 ml), and decrease in absorbance was monitored at 340 nm. Enzyme activity was expressed as nmol of NADPH oxidized/min/mg protein (Horn, 1965).

4.8. Northern blotting

Total RNA from motor cortex and lumbosacral cord of vehicle and L-BOAA treated male and female mice was extracted using TRI reagent (Kenchappa et al., 2002). CNS regions from 3 animals were pooled for each sample preparation. Total RNA was separated electrophoretically and transferred to positively charged nylon membranes by capillary transfer, UV cross-linked and hybridized with digoxigenin-labeled cRNA, prepared using the cDNA to γ-GCS (Kenchappa and Ravindranath, 2003a). The blots were washed, incubated with antibody to digoxigenin conjugated with alkaline phosphatase and bands were visualized by chromogenic substrate for alkaline phosphatase. The relative intensity of the bands was estimated using densitometry.

4.9. Immunohistochemistry

Paraffin embedded sections of mouse cortex and spinal cord were taken and sections were dewaxed. The sections were pressure cooked in sodium citrate buffer (0.01 M, pH 6) for antigen retrieval, blocked with normal goat serum and incubated with antibody of estrogen receptor α and β. Control sections were incubated with non-immune serum. The cortical

sections were washed, treated with biotinylated anti-rabbit IgG and incubated with streptavidin-fluorescein. The spinal cord sections were washed and treated with anti rabbit Alexa-fluor 594. The sections were washed in phosphate buffer saline, dried and mounted.

Statistical analysis was performed using Student's *t* test or ANOVA followed by Student–Newman–Keul's or Dunnett's test as appropriate.

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