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## Aging is associated with dimerization and inactivation of the brain-enriched tyrosine phosphatase STEP

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### Abstract

The striatal-enriched tyrosine phosphatase (STEP) is involved in the etiology of several age-associated neurological disorders linked to oxidative stress and is also known to play a role in neuroprotection by modulating glutamatergic transmission. However, the possible effect of aging on STEP level and activity in the brain is still unclear. In this study, using young (1 month), adult (4 month) and aged (18 month) rats we show that aging is associated with increase in dimerization and loss of activity of STEP. Increased dimerization of STEP is primarily observed in the cortex and hippocampus and is associated with depletion of both reduced and total glutathione levels, suggesting an increase in oxidative stress. Consistent with this interpretation studies in cell culture models of glutathione depletion and oxidative stress also demonstrates formation of dimers and higher order oligomers of STEP that involves intermolecular disulfide bond formation between multiple cysteine residues. Conversely, administration of N-acetyl cysteine, a major antioxidant that enhances glutathione biosynthesis, attenuates STEP dimerization both in the cortex and hippocampus. The findings indicate that loss of this intrinsic protective response pathway with age-dependent increase in oxidative stress may be a contributing factor for the susceptibility of the brain to age-associated neurological disorders.

### Keywords

Tyrosine phosphatase; STEP; dimerization; aging; glutathione; N-acetyl cysteine

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#### Disclosure statement for authors

The authors declare that they have no conflicts of interest.

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## 1. INTRODUCTION

Aging is characterized by a progressive decline in the efficiency of physiological function and by the increased susceptibility to disease and death. Although the fundamental mechanisms are still poorly understood, a growing body of evidence points towards oxidative stress as one of the primary determinants of aging (Hagen, 2003; Kregel and Zhang, 2007). Oxidative stress induced redox imbalance has been associated with aberrant alteration in the tyrosine phosphorylation of key signaling proteins implicated in the pathogenesis of aging and many age-related neurodegenerative disorders (Schmitt et al., 2005; Droge and Schipper, 2007; Jung et al., 2009). The regulation of tyrosine phosphorylation involves agonistic and antagonistic activity of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Although a considerable amount of research has investigated the regulation of PTKs with oxidative stress and aging, our knowledge about the regulation of PTPs with aging is limited (Jin and Saitoh, 1995; Minetti et al., 2002; Schmitt et al., 2005; Wegiel et al., 2011).

The tyrosine phosphatase STEP (STriatal-Enriched Phosphatase) that is expressed exclusively in the central nervous system is emerging as an important regulator of neuronal function (Lombroso et al., 1993; Boulanger et al., 1995). The STEP-family of PTPs includes both membrane associated (STEP<sub>61</sub>) and cytosolic (STEP<sub>46</sub>) variants (Bult et al., 1997). Biochemical and electron microscopic studies have localized STEP<sub>61</sub> to endoplasmic reticulum, mitochondria, synaptic vesicles and post-synaptic densities (Bult et al., 1996). An emerging body of evidence indicates that the alteration in STEP function is associated with the progression of several age-associated acute and chronic neurological disorders including Alzheimer's disease, Parkinson's disorder and cerebral ischemia (Kurup et al., 2010; Deb et al., 2013; Xu et al., 2014; Kurup et al., 2015). An increase in STEP level and activity resulting from a disruption of the proteasomal degradation machinery is thought to interfere with synaptic strengthening resulting in cognitive and behavioral deficits observed in Alzheimer's disease (Kurup et al., 2010). In contrast, lower STEP level and activity due to rapid degradation of the protein has been implicated in ischemia-induced brain damage (Deb et al., 2013). Thus it appears that both high and low level of STEP could disrupt cellular function resulting in neurodegeneration. Despite this evidence for a role of STEP in age-associated neurological disorders, a causal link between STEP and aging has still not been established.

A series of molecular and biochemical studies have found that the activity and stability of STEP is regulated by several post-translational modifications (Paul et al., 2000; Paul et al., 2003; Deb et al., 2011; Mukherjee et al., 2011). Reversible phosphorylation of a regulatory serine residue (Ser 221 in STEP<sub>61</sub>/Ser 49 in STEP<sub>46</sub>) in a conserved domain termed kinase interacting motif (KIM) has been shown to modulate STEP activity in terms of its ability to bind to its substrates (Paul et al., 2000; Paul et al., 2003). Additional studies investigating the regulation of ubiquitin-mediated proteasomal degradation of STEP found that phosphorylation of two SP/TP sites in a second conserved domain termed as kinase specificity sequence are important in maintaining the stability of STEP (Mukherjee et al., 2011). Another potentially important finding is that H<sub>2</sub>O<sub>2</sub> induced oxidative stress leads to significant increase in the oligomerization of STEP in neurons resulting in substantial loss of

its enzymatic activity (Deb et al., 2011). Since aging is associated with an increase in oxidative stress, the present study sought to investigate the effect of aging on STEP dimerization in the brain and its association with the glutathione (GSH) levels, a major antioxidant that functions to maintain intracellular redox equilibrium.

## 2. METHODS

### 2.1. Materials and reagents

Male Sprague-Dawley rats (1, 4 and 18–20 month) were obtained from Harlan Laboratories (Livermore, CA, USA). Antibodies used were as follows: monoclonal anti-STEP from Novus Biologicals (Littleton, CO, USA), monoclonal anti-V5 from Invitrogen (Carlsbad, CA, USA), polyclonal PSD-95 from Cell Signaling, monoclonal anti-cMyc and polyclonal Calnexin from Santa Cruz (Santa Cruz, CA, USA), monoclonal anti- $\beta$ -tubulin and polyclonal synaptophysin from Sigma (St. Louis, MO, USA). All secondary antibodies were from Cell Signaling. Protein G Sepharose was from GE Healthcare. N-acetyl cysteine (NAC), Diethylmaleate (DEM) and *para*-nitrophenylphosphate (pNPP) were from Sigma-Aldrich (St. Louis, MO, USA). GSH assay kit was from Arbor assays (Ann Arbor, MI, USA). All tissue culture reagents were obtained from Invitrogen. Approval for animal experiments was given by the University of New Mexico, Health Sciences Center, Institutional Animal Care and Use Committee.

### 2.2. Detection of dimers and oligomers using non-reducing SDS-PAGE

To detect the dimers and higher order oligomers of STEP both brain tissue and transfected cells were processed as reported previously (Deb et al., 2011). Briefly, cortical, hippocampal and striatal tissues were homogenized in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Nonidet P-40 and cocktail of protease inhibitors, incubated on ice for 45 min and then centrifuged at 13,000 rpm for 10 min. For cell line experiments, cells were washed with PBS and then scraped in the lysis buffer, incubated on ice for 45 min and then centrifuged at 13,000 rpm for 10 min. Equal protein from each sample were diluted 1:1 with 2x Laemmli sample buffer, with  $\beta$ -mercaptoethanol (reducing conditions) or without  $\beta$ -mercaptoethanol (non-reducing conditions). The samples processed under reducing conditions were boiled for 10 min. The reducing and non-reducing samples were separated onto 7.5% or 6% SDS-polyacrylamide gels respectively. The proteins in the gel were transferred to polyvinylidene difluoride membrane and processed for immunoblot analysis.

### 2.3. Immunoblotting

Protein concentration in lysates was estimated using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein from each sample was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. After blocking with 5% non-fat dry milk or BSA, membranes were incubated for 1 hr at room temperature or overnight at 4°C with the appropriate primary antibody. Horseradish peroxidase (HRP) coupled to anti-rabbit or anti-mouse IgG raised in goat were used as secondary antibodies. Immune complexes were detected on X-ray films after treatment with West Pico supersignal chemiluminescence reagents (Thermo Scientific). Densitometric analysis of immunoblots was performed using Image J software (<http://rsbweb.nih.gov/ij/>)

## 2.4. Glutathione assay

Glutathione (GSH) levels in brain homogenates were measured as described previously (Candelario-Jalil et al., 2007) with some modifications. Briefly, cortical, striatal or hippocampal slices were sonicated in PBS (pH 7.0) and then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was processed for protein estimation using BCA kit. Equal amount of protein from each sample (30 µg) was incubated with equal volume of 5% sulfosalicylic acid (SSA) for 10 min in ice, followed by centrifugation at 13,000 rpm for 10 min. The supernatant was then diluted with the supplied assay buffer to bring the concentration of SSA to 1% and then incubated with ThioStar reagent in a black 96-well plate (total volume 50 µl) for 15 min at room temperature to determine the reduced GSH level. Fluorescent signal from each well was quantified spectrofluorometrically (Leica, Buffalo Grove, IL) using excitation and emission wavelengths of 390 and 510 nm, respectively. For quantitation of total GSH level, GSH reductase and NADPH reaction mixture were added to the wells followed by another 15 min incubation at room temperature and spectrofluorometric reading of the signal. Tissue GSH concentration was determined using a GSH standard curve.

To determine GSH levels following DEM or H<sub>2</sub>O<sub>2</sub> treatment, HEK293 cells were washed twice with PBS, scraped with 0.1ml 2.5% SSA, incubated for 10 min on ice and then centrifuged at 13,000 rpm for 10 min (Dasgupta et al., 2005). The supernatant was used for GSH assay as described above. The resulting pellet was dissolved in 1x sample buffer (without β-mercaptoethanol), boiled for 10 min, centrifuged for 10 min at 13000 rpm. The supernatant was processed for protein assay to normalize GSH values against total protein.

## 2.5. Tyrosine phosphatase assay

Cortical tissue was homogenized in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1% NP-40 followed by centrifugation at 13,000 rpm for 10 min to collect supernatant for immunoprecipitation with anti-STEP antibody. Equal amount of protein from each sample (1.5 – 2 mg) was processed for immunoprecipitation with anti-STEP antibody (Deb et al., 2011). The immune complexes bound to protein G beads were then washed three times in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% NP-40 and then once in a buffer containing 30 mM HEPES (pH 7.4) and 120 mM NaCl. For phosphatase assay the beads were incubated for 30 min at 30°C in 100 µl of reaction buffer (30 mM HEPES, pH 6.0, 150 mM NaCl, 10 mM pNPP) and reaction was stopped by addition of 0.9 ml of 0.2 N NaOH. Phosphatase activity of STEP<sub>61</sub> was measured by colorimetric quantitation of the formation p-nitrophenolate at 410 nm using a spectrophotometer (Deb et al., 2011). To assess the amount STEP immunoprecipitated the immune complexes bound to the beads were eluted with SD sample buffer and processed for immunoblot analysis with anti-STEP antibody. For cell line experiments, cells were lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl and 0.1% NP-40, centrifuged at 13,000 rpm for 10 min, collect supernatant for immunoprecipitation with anti-V5 antibody. The samples were then processed for pNPP assay as mentioned above. The values obtained were normalized for the amount of STEP immunoprecipitated (Toledano-Katchalski et al., 2003; Deb et al., 2011).

## 2.6. Immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% NP-40 and protease inhibitor cocktail (Boehringer). Lysates were centrifuged for 10 min at 13,000 rpm to remove insoluble material, and then pre-cleared with protein G sepharose for 1 hr. For immunoprecipitation of STEP, samples were incubated overnight with anti-V5 antibody at 4°C. The immune complexes were incubated with protein G sepharose for 2 hr at 4°C. Beads were collected by centrifugation at 1000 rpm for 2 min and washed three times with lysis buffer. Proteins were eluted using SDS-sample buffer (Laemmli sample buffer with β-mercaptoethanol) and processed for SDS-PAGE and immunoblot analysis with antibodies as described in the individual experiments.

## 2.7. Cell culture and stimulation

Full-length STEP<sub>61</sub>-cDNA was cloned into the pcDNA3.1/Myc-His and pcDNA 3.1/V5-His mammalian expression vectors (Invitrogen). Point mutants of STEP<sub>61</sub> were obtained by polymerase chain reaction (PCR) based site-directed mutagenesis (Stratagene, La Jolla, CA, USA). All mutations were verified by nucleotide sequencing. HEK293 cells obtained from A.T.C.C. were routinely grown in DMEM/F12, supplemented with 10% fetal bovine serum, at 37°C in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air. Cells were transiently transfected with 1 μg DNA using Lipofectamine™ 2000 (Invitrogen). After 24 hr cells were treated with 0 – 50 μM of DEM for 6 hr or 10mM of H<sub>2</sub>O<sub>2</sub> for 5 min in serum free minimal essential medium at 37°C. Cells were then processed for GSH assay, co-immunoprecipitation experiments or total SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis under reducing or non-reducing conditions.

## 2.8. Subcellular fractionation of rat brain tissues

Biochemical fractionation was performed as described previously (Dunah and Standaert, 2001) with minor modifications. Briefly, cortical tissue from 1 old SD rats were homogenized in TEVP buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EDTA, and 1 mM EGTA) containing 320 mM sucrose and was centrifuged for 10 min at 800g to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged for 15 min at 9,200g to obtain the crude synaptosomal fraction (P2). The P2 fraction was re-suspended and incubated in a hypo-osmotic buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EDTA, and 1 mM EGTA) containing 35.6 mM sucrose for 30 min and then centrifuged at 25,000g for 20 min to pellet the synaptosomal membrane fraction (LP1). The resulting supernatant (LS1) was centrifuged for 2 hr at 165,000g to obtain the synaptic vesicle-enriched fraction (LP2). The supernatant S2 was centrifuged at 165,000g for 2 hr to obtain the cytosolic fraction (S3) and a light membrane/microsome-enriched fraction (P3). After each centrifugation the resulting pellet was rinsed briefly with ice-cold TEVP buffer before subsequent fractionations to avoid possible crossover contamination. All centrifugations were performed at 4°C.

## 2.9. Drug treatment

Aged (18–20 month) SD rats were injected intraperitoneally with N-acetyl cysteine (50 mg/kg, dissolved in PBS) daily for two weeks. N-acetyl cysteine (NAC) was dissolved in PBS and made fresh at the beginning of each experiment. Control animals were injected with PBS daily for 2 weeks. 24 hr after the last injection one group of drug treated and control rats were processed for enzymatic evaluation of glutathione (GSH) level in the cortex and hippocampus. Another set of drug treated and control rats were processed for immunoblot analysis under both reducing and non-reducing conditions.

## 3.0. Data Analysis

Data in the text and figures are expressed as mean  $\pm$  SEM. Statistical differences between multiple groups were assessed using one-way ANOVA followed by Bonferroni's *post hoc* comparison test. For statistical differences between two groups analysis was done using Student's *t* test. Differences were considered statistically significant when  $p < 0.05$ .

## 3. RESULTS

### 3.1. Aging is associated with increased dimerization of STEP<sub>61</sub>

To examine the effect of age on dimerization of STEP, cortical, hippocampal and striatal tissues from young (1 month), adult (4 month) and aged (18 month) male SD rats were processed for immunoblot analysis with anti-STEP antibody under non-reducing condition. The results showed an age-dependent migration (upward shift in electrophoretic mobility) of STEP<sub>61</sub> to a position that corresponds to double the molecular weight (100–150 kDa) compared with STEP<sub>61</sub> monomer (61 kDa; Fig. 1, upper panel), suggesting the formation of dimers of STEP<sub>61</sub>. A significant increase in dimerization of STEP<sub>61</sub> was observed only in the cortical (6.2 fold  $\pm$  1.4) and hippocampal (5.9 fold  $\pm$  1.5) tissue homogenates obtained from the aged rats (Fig. 1A, 1B, upper panel). However, the dimerization of STEP in striatal homogenates that expresses both STEP<sub>61</sub> and STEP<sub>46</sub> did not change significantly with age (Fig. 1C, upper panel). Immunoblot analysis of the same samples under reducing conditions (in the presence of  $\beta$ -mercaptoethanol), where disulfide bond formation cannot occur, showed only the monomeric form of STEP (Fig. 1A–C, middle panel). These findings imply that age-associated increase in oxidative stress may play a role in the dimerization of STEP<sub>61</sub>.

### 3.2. Aging is associated with depletion of glutathione level and loss of activity of STEP<sub>61</sub>

Depletion of the endogenous antioxidant, GSH has been associated with moderate to severe oxidative stress (Calabrese et al., 2000; Drake et al., 2003) and several studies have shown that both the reduced and total GSH levels decrease in many aged tissues due to increased GSH consumption and/or decreased GSH production (Maher, 2005; Ballatori et al., 2009; Emir et al., 2011; Currais and Maher, 2013). Therefore, we evaluated both the reduced and total GSH levels in cortical, hippocampal and striatal tissues obtained from young, adult and aged rats. As shown in Figure 2 (A–C) reduced GSH levels in the adult rat brains remained unaltered when compared with the younger rats. However, in the aged rats reduced GSH levels decreased considerably in both the cortex ( $11.6 \pm 0.3$  in the aged rats vs  $17 \pm 0.8$  in

the young) and the hippocampus ( $13.2 \pm 0.3$  in the aged rats vs  $16.7 \pm 0.7$  in the young rats). GSH level also decreased in the striatum of the aged rats but to a lesser extent ( $13.5 \pm 0.3$  in aged rats vs  $14.9 \pm 0.6$  in the young rats) than observed in the cortex and the hippocampus. The data presented in Figure 2 (D–F) further show a substantial decrease in total GSH levels in both the cortex ( $13.5 \pm 0.4$  in aged rats vs  $18.8 \pm 0.9$  in the young rats) and the hippocampus ( $13.8 \pm 0.5$  in aged rats vs  $17.5 \pm 0.8$  in the young rats) of the aged rats, while in the striatum the decrease is less pronounced ( $13.7 \pm 0.2$  in aged rats vs  $15.2 \pm 0.5$  in the young rats). These findings suggest that the depletion of both the reduced and total GSH levels vary in different brain regions and the consequences of GSH depletion on STEP dimerization depends on the GSH level.

To determine whether the increased dimerization of STEP<sub>61</sub> in aged rats has any effect on its phosphatase activity, STEP was immunoprecipitated from the cortex, hippocampus and striatum of young and aged rats. Tyrosine phosphatase activity of STEP<sub>61</sub> was measured using pNPP as a substrate and the values were normalized for the amount of STEP<sub>61</sub> immunoprecipitated. As shown in Figure 3 (A–C) a dramatic decrease in STEP activity was observed in the cortex ( $47.5 \pm 3.8\%$ ) and hippocampus ( $40.7 \pm 12.2\%$ ) of aged rats as compared with the younger controls, while in the striatum no significant change was observed.

### 3.3. Subcellular localization of dimerized STEP<sub>61</sub>

To examine the sub-cellular localization of dimerized STEP<sub>61</sub> cortical lysates from 18-month old aged rats were subjected to biochemical fractionation (Fig. 4A). Initial studies examined the relative purity of each fraction using immunoblot analysis of marker proteins for each sub-cellular compartment (Fig. 4B). Calnexin, a calcium-binding phosphoprotein and an integral membrane protein found in endoplasmic reticulum (David et al., 1993) was highly enriched in the light membrane fraction (Fig. 4B, P3, lane 5) but not detected in the synaptosomal membrane (LP1) or synaptic vesicle-enriched (LP2) fractions. The synaptosomal membrane protein postsynaptic density-95 (PSD-95) was enriched in the crude synaptosomal membrane (P2) and synaptosomal membrane (LP1) fractions (Fig. 4B, lanes 3 and 7). The synaptic vesicle membrane protein, synaptophysin (Devoto and Barnstable, 1987), was highly concentrated in the synaptic vesicle enriched fraction (Fig. 4B, LP2, lane 9). It was also present at lower levels in the light membrane (Fig. 4B, P3, lane 5) and synaptosomal membrane (Fig. 4B, LP1, lane 7) fractions. Analysis of each fraction with anti-STEP antibody showed that STEP<sub>61</sub> is present in the light membrane-enriched fraction (P3), synaptosomal membrane fraction (LP1) as well as the synaptic vesicle enriched fraction (LP2). The sub-cellular fractions where STEP<sub>61</sub> was detected were then subjected to immunoblot analysis under non-reducing conditions. As shown in Figure 4C (lanes 4 and 7) dimerized STEP<sub>61</sub> was present in both the light membrane (P3) and the synaptic vesicle (LP2) enriched fractions.

### 3.4. Diethylmaleate induced glutathione depletion leads to dimerization of STEP<sub>61</sub>

To evaluate more directly the effect of depleting intracellular GSH level on the dimerization of STEP<sub>61</sub>, HEK293 cells expressing V5-tagged STEP<sub>61</sub> were treated with varying concentrations of diethyl maleate (DEM, 6 hr), a GSH depleting agent (Plummer et al.,

1981). Enzymatic evaluation of cellular GSH levels showed a significant dose-dependent decrease in both reduced and total GSH levels (Fig. 5A, B). Immunoblot analysis of DEM treated cell lysates, under non-reducing condition showed a concentration-dependent migration of STEP<sub>61</sub> to positions that correspond to double (between 100–150 kDa) and even higher molecular weights (above 150 kDa) compared with STEP<sub>61</sub> monomer, suggesting the formation of dimers and higher order association (oligomers). However, a significant increase in the formation of dimers and higher order oligomers of STEP<sub>61</sub> was observed only with the highest dose of DEM (Fig. 5C and corresponding bar diagram). To determine whether STEP<sub>61</sub> oligomerization following depletion of intracellular GSH levels involves intermolecular interaction, HEK293 cells expressing both V5- and myc-tagged STEP<sub>61</sub> were treated with varying concentrations of DEM. STEP<sub>61</sub>-V5 was immunoprecipitated using anti-V5 antibody and co-immunoprecipitation of STEP<sub>61</sub>-myc was determined by immunoblotting with anti-myc antibody. As shown in Figure 5D, (upper panel and corresponding bar diagram) a significant increase in co-immunoprecipitation of myc-tagged STEP<sub>61</sub> was observed only with the highest concentration of DEM. Re-probing the blot with anti-V5 antibody (Fig. 5D, middle panel) and immunoblot analysis of the input lysates with anti-myc antibody (Fig. 5D, lower panel) showed equal expression of both STEP<sub>61</sub>-V5 and STEP<sub>61</sub>-myc. To evaluate the effect of STEP<sub>61</sub> dimerization on its phosphatase activity, HEK293 cells expressing V5-tagged STEP<sub>61</sub> were treated with DEM (50  $\mu$ M, 6 hr) followed by pNPP assay on immunoprecipitated STEP<sub>61</sub>. As shown in Figure 5E a dramatic decrease in STEP<sub>61</sub> activity was observed in the presence of DEM ( $48.1 \pm 1.1\%$ ) as compared with the untreated control ( $100 \pm 1.3\%$ ). These findings provide further support for our hypothesis that decrease of cellular GSH level below a certain threshold could lead to significant increase in dimerization and subsequent loss of activity of STEP<sub>61</sub>.

### 3.5. Oxidative stress induced depletion of glutathione levels and dimerization of STEP<sub>61</sub>

Numerous studies involving both animal models of aging and elderly human subjects have suggested that increased reactive oxygen species (ROS) generation with aging is a primary contributing factor for age-associated decrease in total GSH levels (Palomero et al., 2001; Rebrin et al., 2003; Maher, 2005; Emir et al., 2011; Giustarini et al., 2011; Currais and Maher, 2013). To evaluate whether exposure to H<sub>2</sub>O<sub>2</sub>, the most abundant form of ROS (Poon et al., 2004; Adam-Vizi, 2005) could lead to alteration in intracellular GSH level, HEK293 cells treated with H<sub>2</sub>O<sub>2</sub> (10 mM, 5 min) were processed for GSH assay. As shown in Figure 6 (A, B) H<sub>2</sub>O<sub>2</sub> treatment led to a significant decrease in both reduced and total GSH levels. To examine the effect of ROS on STEP<sub>61</sub> dimerization HEK293 cells expressing V5-tagged STEP<sub>61</sub> were treated with H<sub>2</sub>O<sub>2</sub> and processed for immunoblotting analysis under non-reducing conditions. As shown in Figure 6C a significant increase in STEP<sub>61</sub> dimerization was observed in the presence of H<sub>2</sub>O<sub>2</sub>. To confirm that ROS-induced STEP<sub>61</sub> oligomerization involves intermolecular interaction, HEK293 cells co-expressing V5- and myc-tagged STEP<sub>61</sub> were treated with H<sub>2</sub>O<sub>2</sub> and then processed for immunoprecipitation of STEP<sub>61</sub>-V5. As shown in Figure 6D co-immunoprecipitation of myc-tagged STEP<sub>61</sub> increased significantly in the presence of H<sub>2</sub>O<sub>2</sub> as compared with untreated control. Taken together with the findings in Figure 5, it appears that intracellular GSH depletion either

chemically induced by DEM or resulting from oxidative stress caused by H<sub>2</sub>O<sub>2</sub> could lead to dimerization of STEP<sub>61</sub>.

### 3.6. Dimerization of STEP<sub>61</sub> involves multiple cysteine residues

In an earlier study we demonstrated that Cys 65 and Cys 76 present in the unique N-terminal domain of STEP<sub>61</sub> plays a role in the low level of basal dimerization of STEP<sub>61</sub> (Deb et al., 2010). To determine the involvement of these two cysteine residues as well as the redox-sensitive catalytic cysteine residue (Cys 472) in DEM and H<sub>2</sub>O<sub>2</sub> induced dimerization of STEP<sub>61</sub>, we generated three mutants of STEP<sub>61</sub>. In the first mutant Cys 65 and Cys 76 were converted into serine residue (V5- and myc- tagged STEP<sub>61</sub> C65S/C76S), in the second mutant the catalytic cysteine residue was converted to serine (V5- and myc- tagged STEP<sub>61</sub> C472S) and in the third mutant all the three cysteine residues were converted to serine (V5- and myc- tagged STEP<sub>61</sub> C65S/C76S/C472S). HEK293 cells co-expressing the V5- and myc-tagged STEP<sub>61</sub> mutants were treated with DEM or H<sub>2</sub>O<sub>2</sub>. Co-immunoprecipitation experiments showed a significant increase in pull down of myc-tagged STEP<sub>61</sub> C65S/C76S with V5-tagged STEP<sub>61</sub> C65S/C76S following treatment with either DEM (Fig. 7A) or H<sub>2</sub>O<sub>2</sub> (Fig. 7D). A similar increase in interaction was also observed between V5- and myc-tagged STEP<sub>61</sub> C472S (Fig. 7B and E). In contrast, mutation of all three cysteine residues (C65, C76, C472) completely abolished the increase in interaction between V5- and myc-tagged STEP<sub>61</sub> C65S/C76S/C472S in the presence of DEM (Fig. 7C) or H<sub>2</sub>O<sub>2</sub> (Fig. 7E). This lack of intermolecular interaction between V5- and myc-tagged STEP<sub>61</sub> C65S/C76S/C472S suggests a role of Cys 65, Cys 76 as well as Cys 472 in the oligomerization of STEP<sub>61</sub>.

### 3.7. Effect of N-acetyl cysteine on glutathione level and dimerization of STEP<sub>61</sub>

N-acetyl cysteine (NAC), the acetylated variant of the amino acid L-cysteine and a precursor of reduced GSH has been shown to enhance the intracellular biosynthesis of GSH in both *in vitro* and *in vivo* studies (Kelly, 1998; Kanwar and Nehru, 2007; Harvey et al., 2008; Prakash and Kumar, 2009). To determine whether NAC can modulate GSH levels in cortex and hippocampus, the two regions that are most affected by age, 18–20 month old aged rats were treated with NAC (50 μM) or vehicle for two weeks. This was followed by enzymatic evaluation of both reduced and total GSH levels in the cortex and hippocampus. As shown in Figure 8 (A, C) a significant increase in reduced GSH level was observed in both the cortex (13.8 ± 0.1 in the NAC treated vs 11.1 ± 0.2 in the untreated rats) and the hippocampus (14.9 ± 0.2 in the NAC treated vs 12.7 ± 0.3 in the untreated rats) following NAC treatment. Total GSH level also increased significantly in both cortex (14.3 ± 0.2 in the NAC treated vs 13.2 ± 0.2 in the untreated rats) and the hippocampus (17.1 ± 0.1 in the NAC treated vs 13.9 ± 0.3 in the untreated rats) but to a lesser extent in the cortex (Fig. 8B, D). In a parallel series of experiment NAC treated rats were processed for immunoblot analysis of cortical and hippocampal lysates under non-reducing conditions to determine the extent of STEP<sub>61</sub> dimerization. As shown in Figure 8 (E, F) NAC treatment resulted in a significant decrease in STEP<sub>61</sub> dimerization in both the cortex (59.4% ± 3.4 of control) and the hippocampus (58.9% ± 6.7 of control). Taken together the findings suggest that modulation of endogenous GSH levels can regulate STEP<sub>61</sub> dimerization.

## 4. DISCUSSION

A key finding of the current study is that depletion of endogenous GSH level with aging is associated with significant increase in dimerization of STEP<sub>61</sub> resulting in substantial loss of phosphatase activity. Increased dimerization of STEP<sub>61</sub> is particularly evident in the cortex and hippocampus of aged animals. Consistent with these findings *ex vivo* studies in HEK293 cells show that depletion of intracellular GSH level either with DEM or H<sub>2</sub>O<sub>2</sub> also increase the formation of dimers and higher order oligomers of STEP<sub>61</sub> that involve intermolecular disulfide bond formation between multiple cysteine residues. Complementary studies in aged animals further show that increase in endogenous GSH levels by NAC supplementation reduces STEP<sub>61</sub> dimerization. These findings demonstrate an important mechanism of regulation of STEP<sub>61</sub> function *in vivo*.

Alteration in redox homeostasis is thought to be the largest risk factor for age-associated decline in cognitive and motor functions and development of both acute and chronic neurological disorders (Yankner et al., 2008; Hekimi et al., 2011). A large number of studies in both rodents and humans have shown age-associated decline in total GSH and/or reduced GSH level in the brain (Ballatori et al., 2009; Emir et al., 2011; Currais and Maher, 2013). However reduction in GSH level with aging does not necessarily involve a total loss of homeostasis but result in a shift towards more oxidative conditions. Such a pro-oxidative shift may not be always associated with a pathological condition but may disrupt redox-associated pro-survival signaling pathways, increasing the susceptibility of the aging brain to functional and metabolic stressors (Currais and Maher, 2013). Consistent with this interpretation our *in vivo* studies show that depletion of GSH level in the aging brain is associated with increased dimerization and inactivation of the brain-specific tyrosine phosphatase STEP that is known to be involved in neuroprotection (Poddar et al., 2010; Deb et al., 2013). The ability of DEM and H<sub>2</sub>O<sub>2</sub> to increase STEP<sub>61</sub> dimerization in the *ex vivo* studies as well as the efficacy of NAC supplementation to reduce STEP<sub>61</sub> dimerization *in vivo* validates the role of GSH depletion in STEP<sub>61</sub> dimerization.

Another important finding of the present study is that although GSH level decreases significantly in the cortex, hippocampus and striatum the dimerization of STEP<sub>61</sub> does not increase significantly in all the three regions. A substantial increase in the dimerization of STEP<sub>61</sub> is observed primarily in the cortex and hippocampus and to a much lesser extent in the striatum of the aged animals. This implies that the consequences of GSH depletion depend largely upon the cellular level of GSH. Thus, a significant but relatively small depletion of GSH as observed in the striatum fails to alter the dimerization of STEP<sub>61</sub>, while higher levels of GSH loss in the cortex and hippocampus leads to enhanced STEP<sub>61</sub> dimerization. The findings from DEM dose-kinetics study in HEK293 cells, demonstrating increased dimerization of STEP<sub>61</sub> with only the highest dose of DEM in spite of a significant depletion of GSH with all the doses of DEM tested, further support this hypothesis. Such graded response following depletion of GSH level has also been reported in an earlier study, demonstrating exponential increase in ROS generation once GSH level falls below 20% of control values (Maher, 2005). In our studies this exponential increase in ROS generation could account for the very large increase in STEP<sub>61</sub> dimerization observed in the cortex and hippocampus of aged animals and in the DEM treated cells, where GSH

levels fall below 20% of control values. Consistent with this view the data obtained from NAC treated animals also indicate that a ~20% increase in reduced GSH level is sufficient to limit the exponential increase in ROS resulting in substantial decrease STEP<sub>61</sub> dimerization in both cortex and hippocampus. Total GSH level also increases significantly in both these regions but to a lesser extent in the cortex. This could be attributed to the different metabolic conditions of the phenotypically distinct neuronal sub-population in the two regions that may result in variable uptake of NAC and synthesis of glutathione (Braak and Braak, 1994; Conrad et al., 2004; Reznikov et al., 2008). In comparison to the cortex and hippocampus, the relatively small depletion of GSH level in the striatum suggests the presence of compensatory pathways to help maintain GSH level in the aging striatum. In this context, an earlier study investigating the global gene expression profile in the cortex, hippocampus, striatum, cerebellum and spinal cord of young and aged rats observed the fewest age related changes in gene expression in the striatum and the cerebellum (Xu et al., 2007). Their findings also showed that decrease in the expression of genes related to mitochondrial function is less pronounced in the striatum and cerebellum as compared to the other three regions. This would imply a relatively efficient mitochondrial metabolic capacity and function in these two regions, which in turn could alleviate oxidative stress and GSH depletion with aging. Taken together these findings indicate region-specific changes in oxidative stress in the brain during aging, with the cortex and hippocampus being more susceptible than the striatum. Further support for this hypothesis comes from earlier studies demonstrating Ca<sup>2+</sup> dysregulation and mitochondrial dysfunction in the cerebral cortex and hippocampus of the aging brain, two phenomenon that are considered direct causes of increased oxidative stress (Hartmann et al., 1996; Brown et al., 2004; Navarro et al., 2008; Navarro et al., 2011). Additional studies have also shown that both cerebral cortex and hippocampus undergo metabolic and morphological atrophy during aging in both rats and humans, which is also suggestive of oxidative damage (De Leon et al., 1997; Fjell et al., 2009).

Previous studies have indicated that oxidative stress can lead to selective oxidation of cysteine residues to sulfenic acid in a variety of proteins that includes both kinases and phosphatases, and some cysteine residues are more susceptible to oxidation than others because of their lower pKa (Paulsen and Carroll, 2010). Oxidation of such redox sensitive cysteine residues is transient as they quickly undergo further oxidation to sulfinic acid and sulfonic acid or in some cases rapidly reacts with amino acid residues in the proximity to generate secondary products. These include the formation of sulphenylamides by reaction with a neighboring peptide backbone residue and intramolecular disulfides by interacting with a proximal cysteine residue (Lo Conte and Carroll, 2013). Several *in vitro* studies have shown that such oxidative modifications of the redox sensitive catalytic cysteine residue in certain PTPs such as PTP1B, SHP-1 and SHP-2 plays a role in the regulation of their activity (Ostman et al., 2011; Lo Conte and Carroll, 2013). However, dimerization of native PTPs involving intermolecular disulfide bond formation between cysteine residues, during oxidative stress or aging, has been difficult to observe, and has mostly been reported using chimeric proteins and chemical cross-linkers in *in vitro* studies (Desai et al., 1993; Jiang et al., 1999; Walchli et al., 2005; Lee et al., 2007). In this context dimerization of STEP<sub>61</sub> is unique as it is readily detectable both *in vivo* as well as in cells overexpressing STEP<sub>61</sub>

suggesting that homodimerization is an inherent property of STEP<sub>61</sub>. The increased dimerization of STEP<sub>61</sub> in the microsomal and synaptic vesicle enriched fractions further indicates a shift in intracellular redox state at specific subcellular microdomains in the aged brain. The three cysteine residues involved in this aberrant increase in dimerization of STEP<sub>61</sub> involves the redox sensitive catalytic cysteine residue in the highly conserved phosphatase domain as well as Cys 65 and Cys 76 located in the unique N-terminal domain of STEP<sub>61</sub>. The involvement of Cys 65 and Cys 76 in a low level of basal dimerization of STEP<sub>61</sub> in the absence of any stimulation has also been reported in an earlier study (Deb et al., 2011). Together, these findings indicate that the degree of oxidative stress determines the extent of STEP dimerization as well as the differential involvement of the cysteine residues in the intermolecular disulfide bond formation. Since the monomeric form of STEP is necessary for substrate binding, enzymatic activity and neuroprotective function (Deb et al., 2013), such inactivation of STEP through dimerization in the aging brain may facilitate nerve cell damage by promoting increased tyrosine phosphorylation of its physiological substrates that includes both extracellular signal-related kinase (ERK) and p38 mitogen activated protein (MAP) kinases as well as the Src-family of non-receptor tyrosine kinases.

Several studies that sought to characterize the effect of GSH depletion on intracellular signaling have shown that disruption of de novo GSH synthesis or induction of oxidative stress could trigger the activation of p38 MAP kinase (McLaughlin et al., 2001; Filomeni et al., 2003; Zhang et al., 2004; Zhang et al., 2007; Filomeni et al., 2012). Activation of p38 MAP kinase has also been identified in neurons exposed to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), neurotoxins that triggers ROS production and are implicated in the mechanism of cell death (Du et al., 2001; Choi et al., 2004). *In vivo* studies in animal models of vascular dementia also showed activation of p38 MAP kinase in the hippocampus resulting in neurological dysfunction (Yang et al., 2013). Similarly a growing number of studies have established a role of ERK MAP kinase in neuronal injury following depletion of intracellular GSH level or induction of oxidative stress by H<sub>2</sub>O<sub>2</sub> (Du et al., 2002; de Bernardo et al., 2004; Luo et al., 2007; Numakawa et al., 2007; Tuerxun et al., 2010). Oxidative stress caused by prolonged exposure to glutamate (5 mM) also leads to sustained activation of ERK MAP kinase that leads to caspase dependent cell death (Stanciu et al., 2000). In several studies exogenous addition of NAC has been shown to prevent activation of MAP kinases, which is consistent with a role of GSH in regulating MAP kinase function ((Yu et al., 2000; Limon-Pacheco et al., 2007). In addition to their effects on MAPKs, GSH depleting agents as well as oxidative stress inducers have also been shown to trigger activation of Src kinase (Abe et al., 2000; Yoshizumi et al., 2000; Esposito et al., 2003; McCord and Aizenman, 2014). Thus it appears that the changes in redox state in the aging brain with lowering of GSH levels may result in the activation of several kinases that are substrates of STEP. The impairment of STEP that normally functions to limit the duration of activation of ERK MAP kinase, p38 MAP kinase and Src-mediated signaling could therefore facilitate the prolonged activation of these kinases and is likely to have many unintended sequences in the aging brain.

In conclusion, the present study provides the first evidence of age-associated increased dimerization and subsequent loss of phosphatase activity of a PTP in the brain. The findings also provide new insights into the signaling mechanisms underlying oxidative stress during

aging and suggest that loss of function of STEP with aging could play a role in accelerating the progression of age-associated neurodegenerative disorders. Based on these findings subsequent studies will assess the role of STEP in modulating adaptive responses and pro-apoptotic pathways to further our understanding of the function of STEP in age-associated neurodegenerative disorders related to oxidative stress.

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## Abbreviations

<b>STEP</b>	striatal-enriched phosphatase
<b>PTP</b>	protein tyrosine phosphatase
<b>p38 MAPK</b>	p38 mitogen-activated protein kinase
<b>ERK MAPK</b>	extracellular-regulated kinases/mitogen-activated protein kinase
<b>GSH</b>	glutathione
<b>pNPP</b>	<i>para</i> -nitrophenylphosphate
<b>SDS</b>	sodium dodecyl sulfate
<b>DEM</b>	Diethylmaleate

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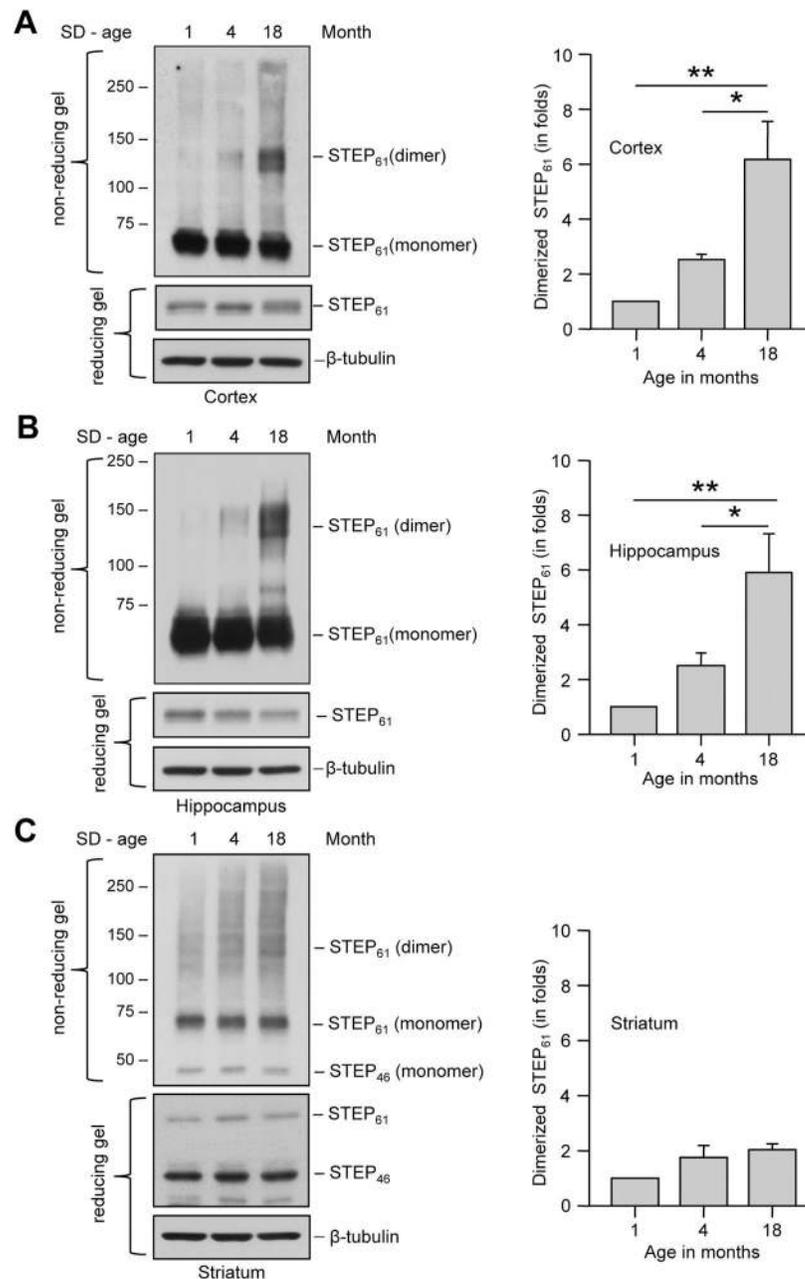
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### Highlights

The most important findings of the current manuscript are:

- Aging is associated with increased dimerization and loss of intrinsic phosphatase activity of the brain-enriched tyrosine phosphatase STEP.
- Increased dimerization and loss of phosphatase activity of STEP is primarily observed in the cortex and hippocampus, and strongly correlates with depletion of total glutathione level.
- Administration of N-acetyl cysteine reduces STEP dimerization both in cortex and hippocampus.
- The formation of dimers and higher order oligomers of STEP are also observed in cultured cells following glutathione depletion either chemically induced by diethylmaleate or H<sub>2</sub>O<sub>2</sub> induced oxidative stress.
- Dimerization of STEP involves intermolecular disulfide bond formation involving two cysteine residues in the unique N-terminal domain and the catalytic cysteine residue.



**Figure 1.**

Aging leads to increased dimerization of STEP in cortex and hippocampus. Equal amounts of protein from (A) cortical, (B) hippocampal and (C) striatal lysates obtained from 1, 4 and 18 month old male SD rats were processed for gel electrophoresis under non-reducing conditions (without  $\beta$ -mercaptoethanol) followed by immunoblotting with anti-STEP antibody to evaluate STEP dimer formation (upper panel). Equal amounts of protein from the same samples were processed under reducing conditions (with  $\beta$ -mercaptoethanol) to assess STEP expression level (middle panel) and the blots were re-probed with anti-tubulin antibody to indicate equal protein loading (lower panel). (A–C) Quantification of dimeric

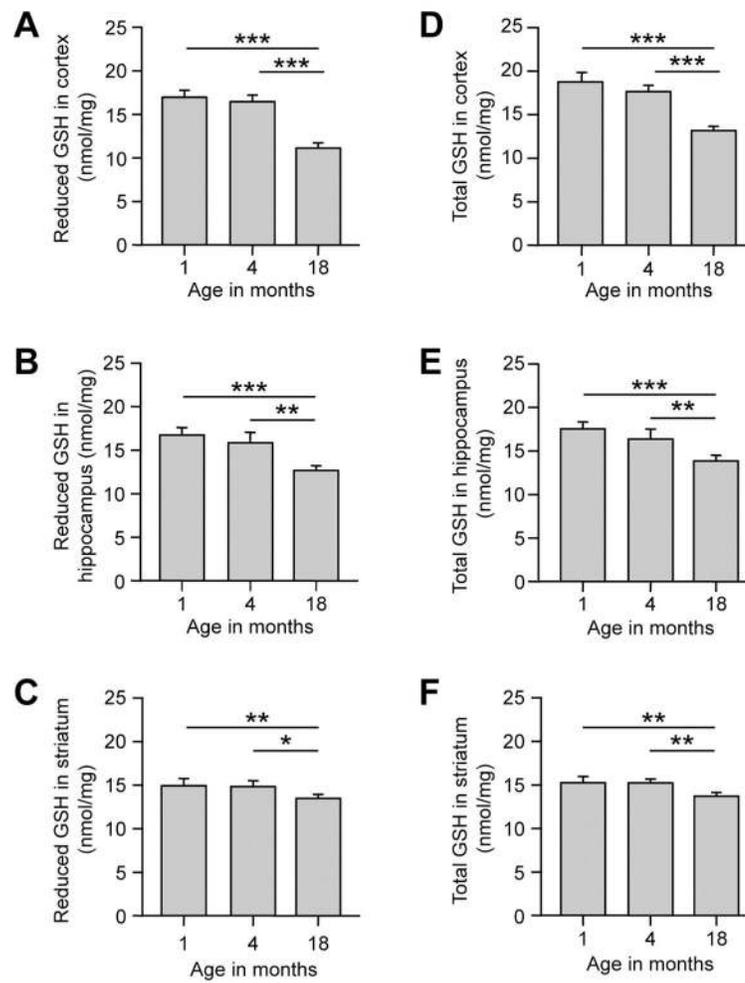
STEP<sub>61</sub> in non-reducing gels (band at 100–150 kDa) was performed by computer-assisted densitometry and image J analysis (right column). Values are mean ± SEM (n = 5). \*p < 0.05, \*\*p < 0.01.

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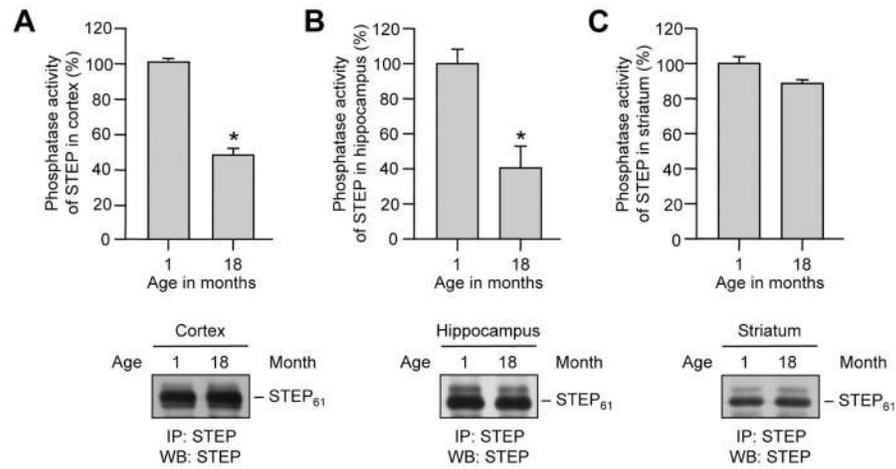
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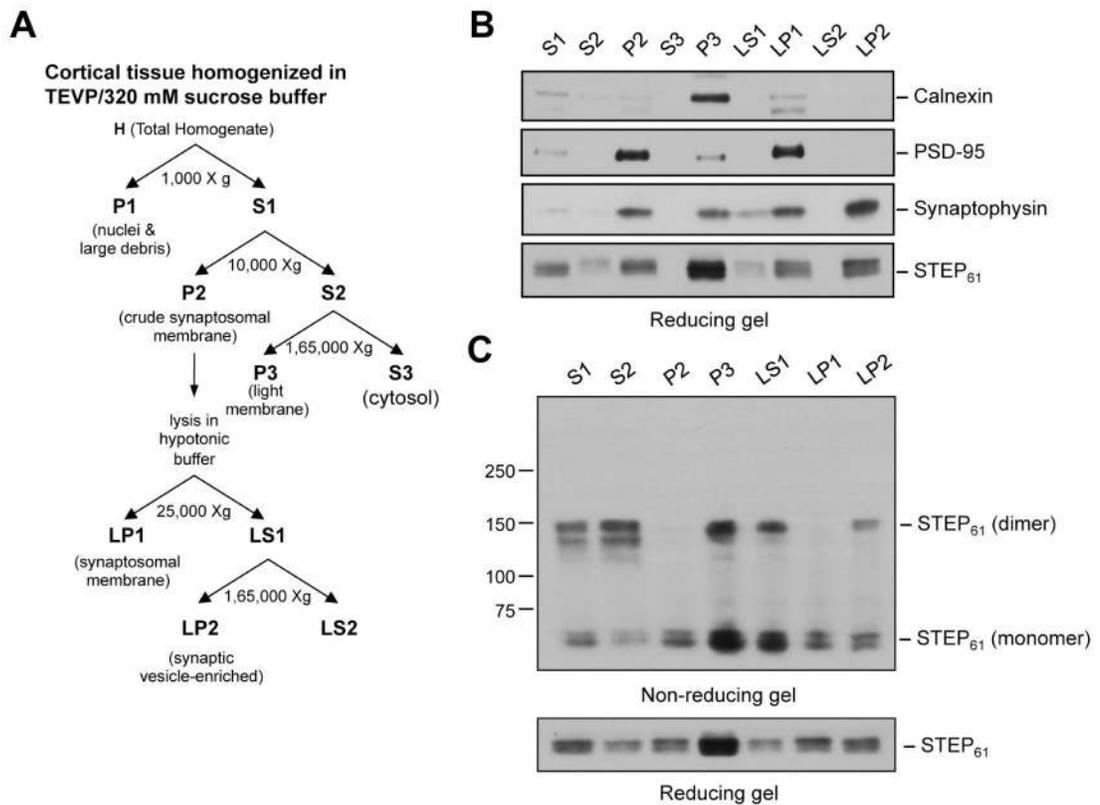
**Figure 2.**

Aging is associated with depletion of both reduced and total GSH levels. (A, D) Cortical, (B, E) hippocampal and (C, F) striatal lysates obtained from 1, 4 and 18 month old SD rats were processed for quantification of both reduced (A–C) and total (D–F) GSH levels. The fluorescent signal generated from the ThioStar-GSH adduct was measured fluorometrically (Ex/Em=390/510 nm). Bar diagrams represent mean  $\pm$  SEM of reduced and total GSH levels ( $n = 5$ ). \* $p < 0.05$ ; \*\* $p < 0.005$ ; \*\*\* $p < 0.0001$ .

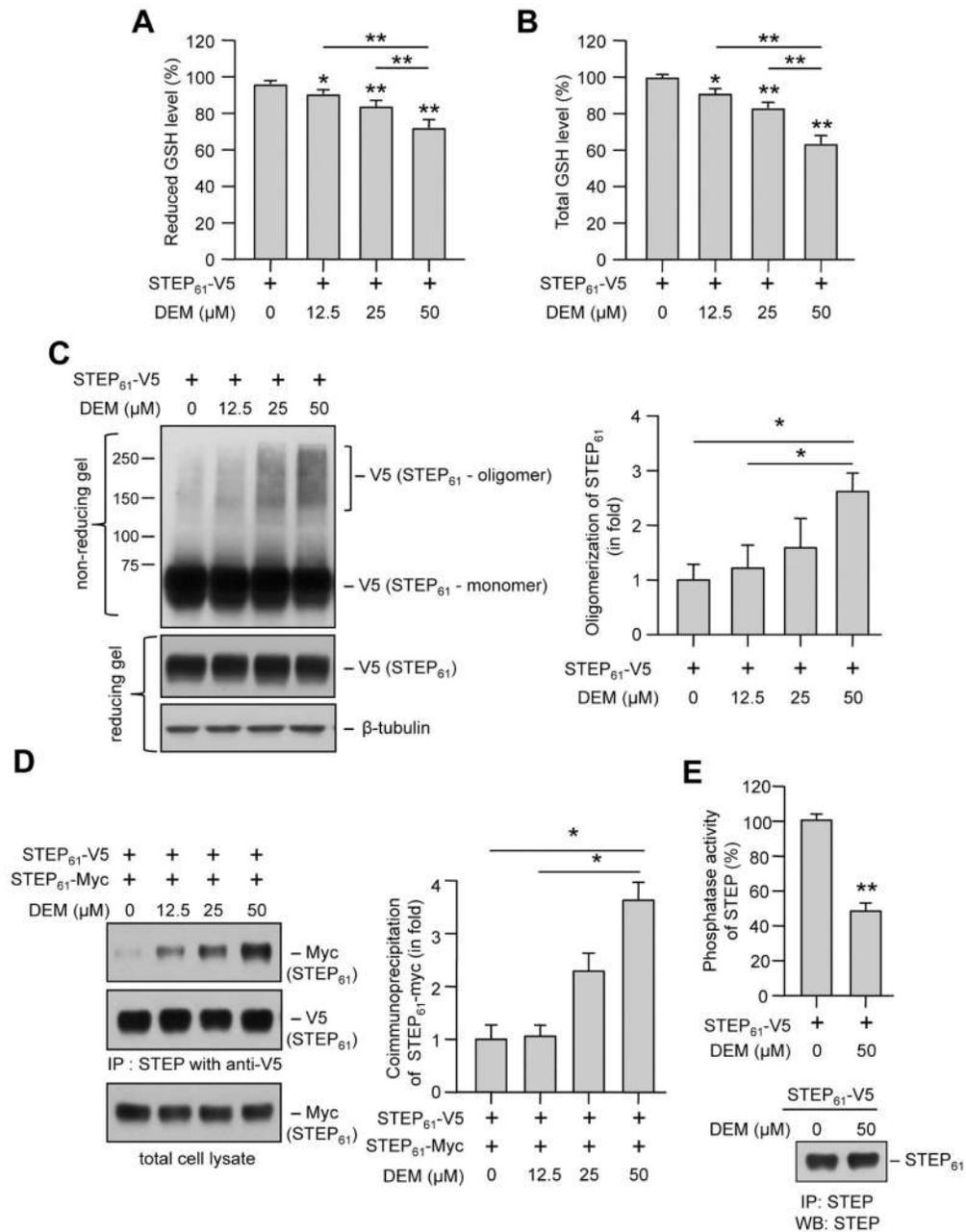


**Figure 3.**

Aging is associated with decrease in STEP phosphatase activity. (A) Cortical, (B) hippocampal and (C) striatal lysates obtained from 1 and 18 month old SD rats were processed for immunoprecipitation of STEP using anti-STEP antibody. PTP activity was assayed using pNPP as a substrate. Quantitative measurement of the formation of para-nitrophenolate is represented as mean  $\pm$  SEM (n = 4). \*p < 0.01. In a parallel series of experiments, immunoprecipitated STEP was subjected to immunoblotting with anti-STEP antibody. Representative immunoblots show equal pull down of STEP.

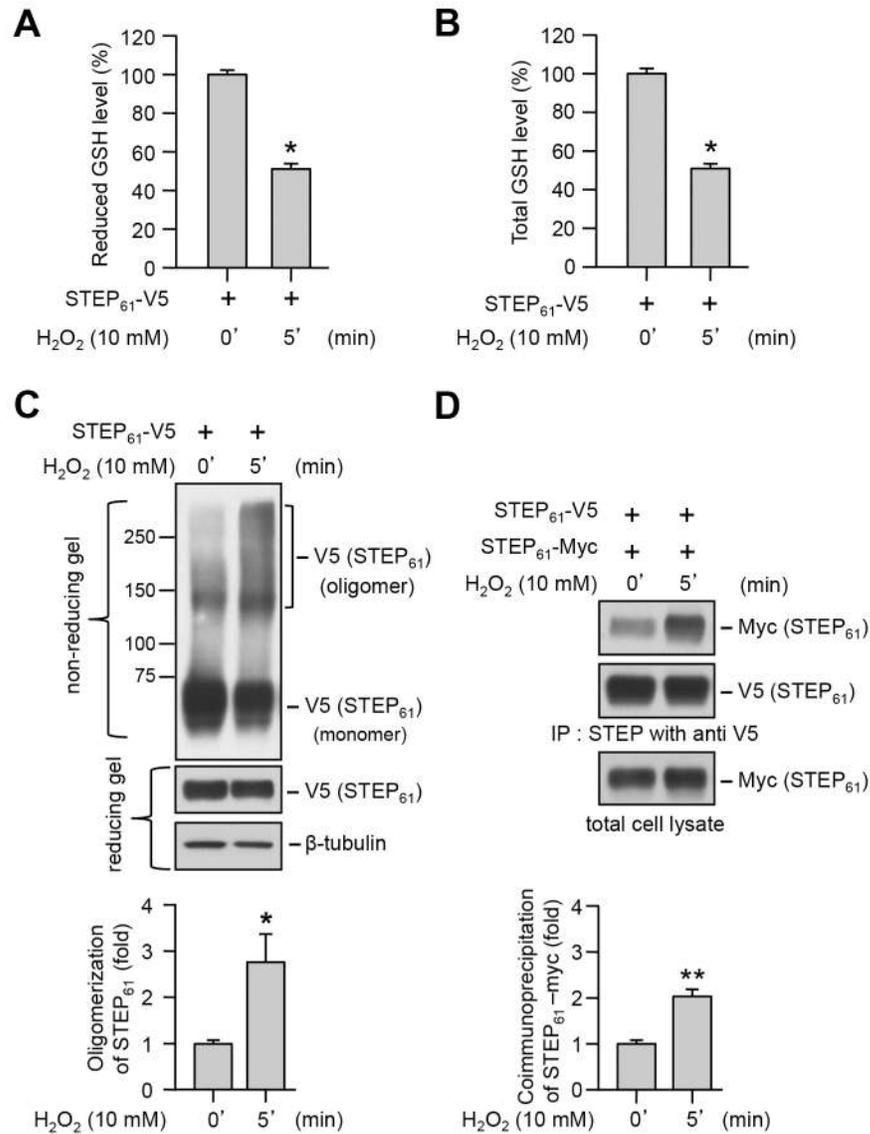


**Figure 4.** Sub-cellular distribution of dimerized STEP<sub>61</sub> in the cortex. (A) Schematic representation of biochemical fractionation of the sub-cellular compartments in cortical homogenates. (B) Equal amounts of protein from each of the isolated biochemical fractions was processed for immunoblotting (reducing condition) with anti-calnexin (panel 1), -PSD-95 (panel 2) and -synaptophysin (panel 3) antibodies to evaluate the purity of each fraction. The sub-cellular distribution of STEP<sub>61</sub> in each of these fractions was evaluated using anti-STEP antibody (panel 4). (C) Protein extracts from the STEP-containing fractions were processed under non-reducing conditions to examine STEP dimer formation, using anti-STEP antibody (upper panel). The same samples were analyzed under reducing conditions to assess total STEP level (lower panel).



**Figure 5.** DEM induced depletion of GSH levels and oligomerization of STEP<sub>61</sub>. HEK293 cells expressing (A–C, E) V5-tagged STEP<sub>61</sub> or (D) V5- and myc- tagged STEP<sub>61</sub> were stimulated with the specified concentrations of DEM for 6 hr. (A, B) Cell lysates were processed for quantitation of reduced and total GSH levels. Bar diagram represents mean ± SEM (n = 4) of fluorescent signal in GSH assay (C) Immunoblot analysis of protein extracts from each sample under non-reducing conditions showing dose-dependent changes in STEP oligomer formation (upper panel). The same samples were analyzed under reducing conditions to assess STEP expression level (middle panel) and blots were re-probed with anti-tubulin antibody to indicate equal protein loading (lower panel). Bar diagram represent

mean  $\pm$  SEM (n = 4) of oligomerized STEP<sub>61</sub> in non-reducing gels. (D) V5-tagged STEP<sub>61</sub> was immunoprecipitated with anti-V5 antibody and co-immunoprecipitation of myc-tagged STEP<sub>61</sub> was determined by probing with anti-myc antibody (upper panel). The blots were re-probed with anti-V5 antibody (middle panel). Expression of myc-tagged STEP<sub>61</sub> in total lysates was analyzed using anti-myc antibody (lower panel). Bar diagram represent mean  $\pm$  SEM (n = 4) of co-immunoprecipitated myc-tagged STEP<sub>61</sub>. (E) Phosphatase activity of STEP<sub>61</sub> immunoprecipitated from HEK293 cells following treatment with DEM was assessed using pNPP as a substrate. Bar diagram represents mean  $\pm$  SEM (n = 5) of STEP phosphatase activity. In a parallel series of experiments immunoprecipitated STEP<sub>61</sub> was processed for immunoblotting with anti-STEP antibody. Representative immunoblot show equal pull down of STEP. \*p < 0.02, \*\*p < 0.0001.

**Figure 6.**

Oxidative stress induced depletion of GSH levels and oligomerization of STEP<sub>61</sub>. HEK293 cells expressing (A–C) V5-tagged STEP<sub>61</sub> or (D) V5- and myc- tagged STEP<sub>61</sub> were stimulated with H<sub>2</sub>O<sub>2</sub> for 5 min. (A, B) Cell lysates were processed for quantitation of both reduced and total GSH levels. Bar diagram represents mean ± SEM (n = 4) of fluorescent signal in GSH assay. (C) Immunoblot analysis of protein extracts under non-reducing (upper panel) and reducing (middle panel) conditions to assess STEP oligomerization and expression level. Immunoblots processed under reducing conditions were re-probed with anti-tubulin antibody (lower panel). Bar diagram represent mean ± SEM (n = 4–5) of oligomerized STEP<sub>61</sub> in non-reducing gels. (D) V5-tagged STEP<sub>61</sub> was immunoprecipitated with anti-V5 antibody and co-immunoprecipitation of myc-tagged STEP<sub>61</sub> was determined by probing with anti-myc antibody (upper panel). The blots were re-probed with anti-V5 antibody (middle panel). Expression of myc-tagged STEP<sub>61</sub> in total lysates was analyzed

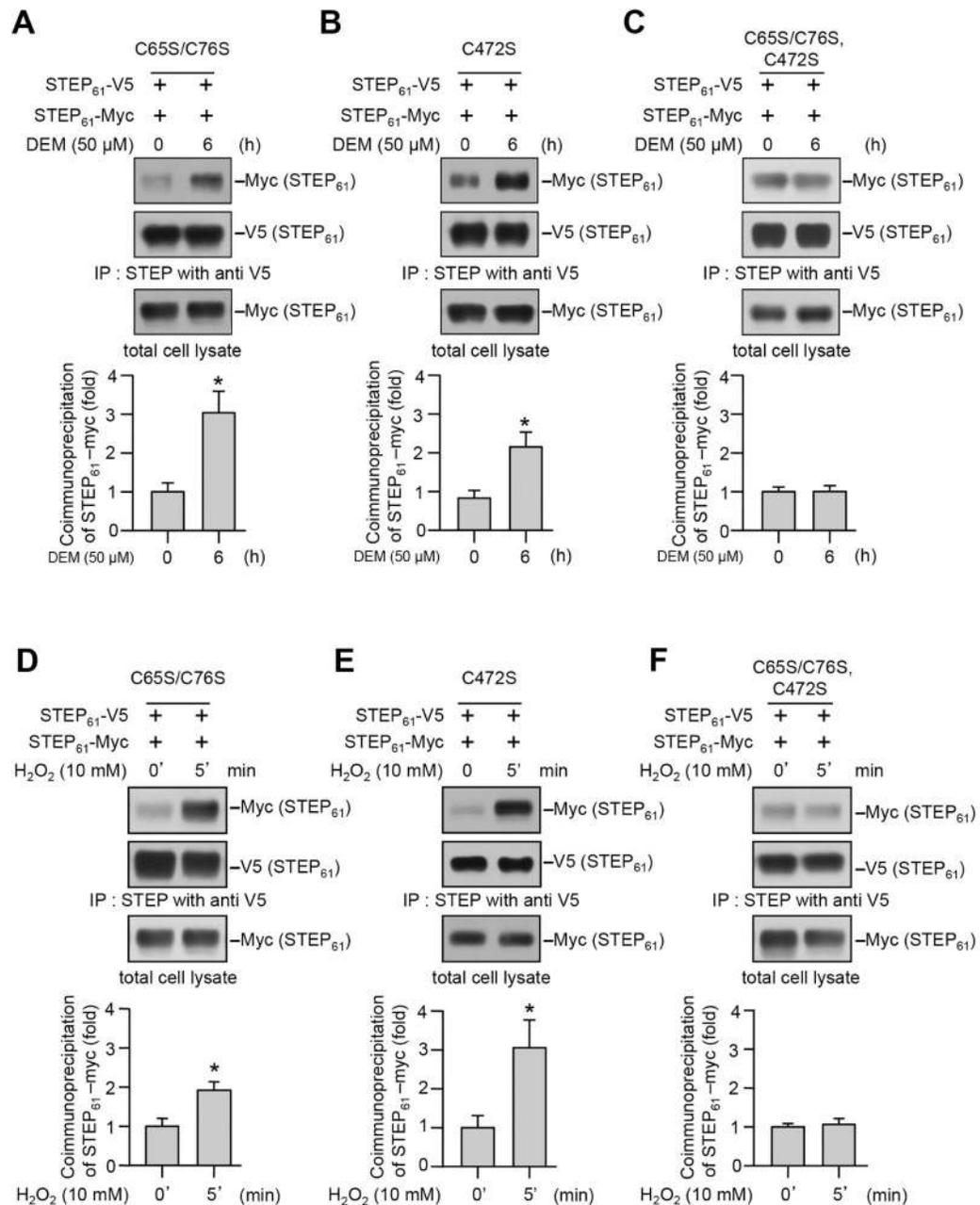
using anti-myc antibody (lower panel). Bar diagram represent mean  $\pm$  SEM of co-immunoprecipitated myc-tagged STEP<sub>61</sub>. \*p < 0.05, \*\*p < 0.01.

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**Figure 7.**

Involvement of multiple cysteine residues in DEM and H<sub>2</sub>O<sub>2</sub> induced dimerization of STEP<sub>61</sub>. HEK293 cells expressing V5- and myc-tagged (A, D) STEP<sub>61</sub> C65S/C76S, (B, E) STEP<sub>61</sub> C472S or (C, F) STEP<sub>61</sub> C65S/C76S/C472S were treated with (A–C) DEM for 6 hr or (D–F) H<sub>2</sub>O<sub>2</sub> for 5 min. V5-tagged STEP<sub>61</sub> was immunoprecipitated using anti-V5 antibody and co-immunoprecipitation of myc-tagged STEP<sub>61</sub> was determined by probing the blots with anti-myc (upper panels) antibody. The blots were re-probed with anti-V5 antibody (middle panels). Expression of myc-tagged STEP<sub>61</sub> in total lysates was analyzed

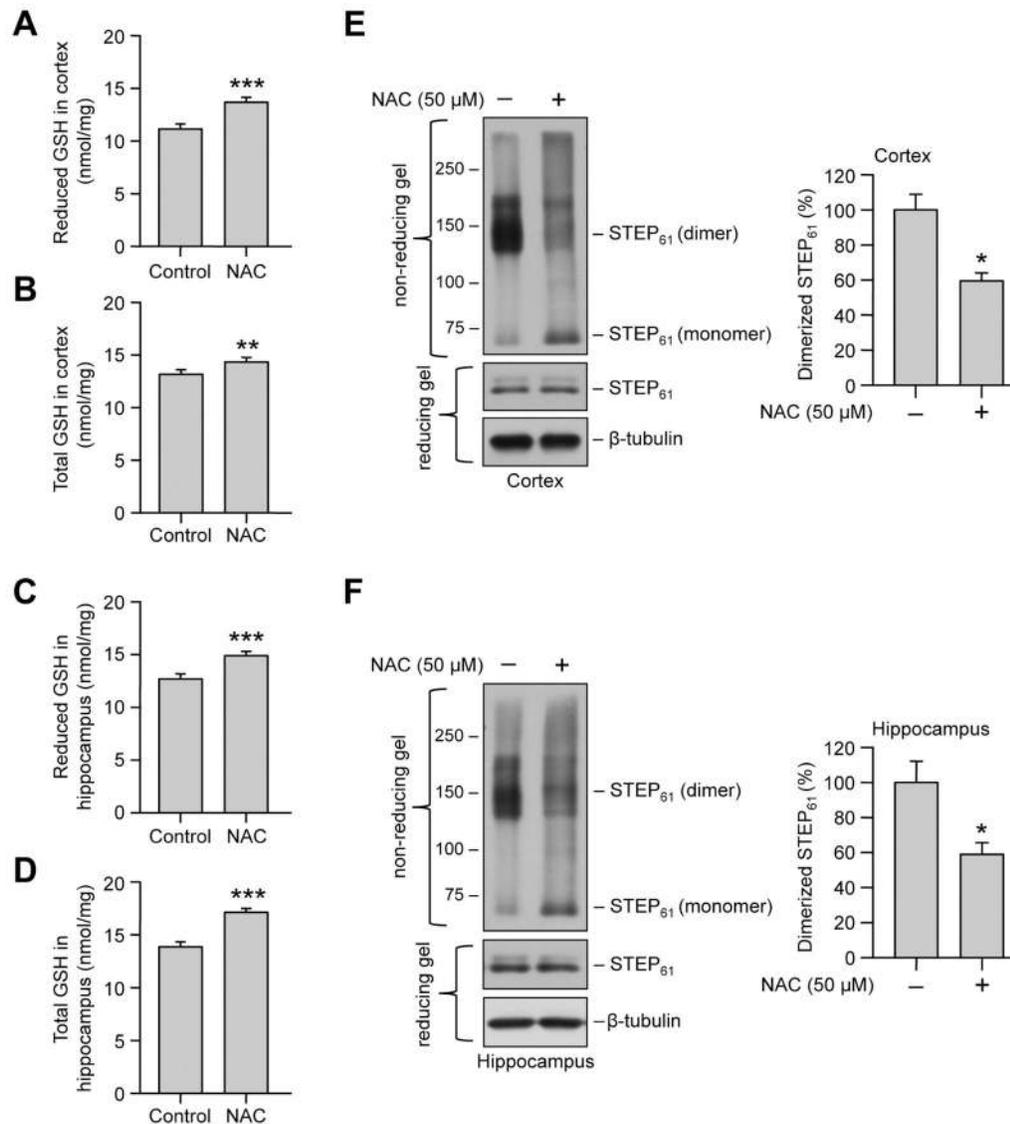
using anti-myc antibody (lower panels). Bar diagram represent mean  $\pm$  SEM (n = 4–5) of co-immunoprecipitated myc-tagged STEP<sub>61</sub>. \*p < 0.05.

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**Figure 8.** Effect of N-acetyl cysteine on GSH levels and STEP<sub>61</sub> dimerization in cortex and hippocampus of aged rats. (A, B) Cortical and (C, D) hippocampal lysates from NAC treated rats were processed for quantification of both reduced and total GSH levels. Bar diagrams represent mean  $\pm$  SEM of reduced and total GSH levels (n = 5). (E, F) In a parallel series of experiments cortical and hippocampal lysates treated with NAC were processed for immunoblot analysis under non-reducing conditions to assess STEP dimerization (upper panels). The same samples were processed under reducing conditions to assess STEP expression level (middle panels) and the blots were re-probed with anti-tubulin antibody (lower panels). Bar diagram represent mean  $\pm$  SEM of dimerized STEP<sub>61</sub> in non-reducing gels (n = 5). \*p < 0.02, \*\*p < 0.005; \*\*\*indicates p < 0.005.