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Role of antioxidant enzymes in redox regulation of NMDAR function and memory in middle-age rats

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Abstract

Overexpression of superoxide dismutase 1 (SOD1) in the hippocampus results in age-dependent impaired cognition and altered synaptic plasticity suggesting a possible model for examining the role of oxidative stress in senescent neurophysiology. However, it is unclear if SOD1 overexpression involves an altered redox environment and a decrease in N-methyl-D-aspartate receptor (NMDAR) synaptic function reported for aging animals. Viral vectors were used to express SOD1 and green fluorescent protein (SOD1+GFP), SOD1 and catalase (SOD1+CAT), or GFP alone in the hippocampus of middle-age (17 mo) male Fischer 344 rats. We confirm that SOD1+GFP and SOD1+CAT reduced lipid peroxidation indicating superoxide metabolites were primarily responsible for lipid peroxidation. SOD1+GFP impaired learning, decreased glutathione peroxidase (GPx) activity, decreased glutathione (GSH) levels, decreased NMDAR-mediated synaptic responses, and impaired long-term potentiation (LTP). Co-expression of SOD1+CAT rescued the effects of SOD1 expression on learning, redox measures, and synaptic function suggesting the effects were mediated by excess hydrogen peroxide. Application of the reducing agent dithiothreitol (DTT) to hippocampal slices increased the NMDAR-mediated component of the synaptic response in SOD1+GFP animals relative to animals that overexpress SOD1+CAT indicating that the effect of antioxidant enzyme expression on NMDAR function was due to a shift in the redox environment. The results suggest that overexpression of neuronal SOD1 and CAT in middle-age may provide a model for examining the role of oxidative stress in senescent physiology and the progression of age-related neurodegenerative diseases.

Keywords

Aging; superoxide dismutase; catalase; glutathione peroxidase; learning and memory; NMDAR; synaptic plasticity

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1. Introduction

The oxidative stress theory of aging suggests that treatments which enhance or reduce oxidative stress/damage will have predictable effects in promoting or delaying aging processes. This theory has come under criticism due to the failure of overexpressed antioxidant genes to extend lifespan [35]. Rather, antioxidant genes appear to influence age-related changes in function or physiology, and the trajectory or progression of pathology in a manner predicted by the oxidative stress theory of aging [39]. In the case of brain aging and cognitive decline, oxidative damage likely contributes to cell death observed during the progression of neurodegenerative disease; however, recent work suggests that a change in the oxidation-reduction (redox) status of the intracellular environment may underlie senescent neurophysiology and the earliest signs of cognitive aging [5,6,20,22,30,38]. However, studies that point to the redox environment in altering memory mechanisms are usually correlative in nature, describing redox-mediated changes during aging rather than directly modifying oxidative stress. Thus, recent work demonstrates that Ca^{2+} dysregulation and senescent synaptic function in the hippocampus during aging is due to an oxidized intracellular redox environment [4,6,17]. In particular, redox changes mediate an age-related decrease in N-methyl-D-aspartate receptor (NMDAR) synaptic responses and impaired NMDAR-dependent long-term potentiation (LTP) [5,22,38]. Furthermore, the decline in NMDAR function emerges in middle-age specifically in animals that exhibit impaired spatial episodic memory [28]. However, no study has explicitly tested the prediction that overexpressed antioxidant genes will modify NMDAR function in a manner consistent with the idea that the redox environment determines the extent of senescent neurophysiology.

One potential means for testing this idea is through the overexpression of superoxide dismutase 1 (SOD1). To prevent oxidative damage, SOD1 catalyzes the conversion of superoxide into less active hydrogen peroxide. However, the relatively milder hydrogen peroxide can induce a shift in the intracellular oxidation-reduction (redox) environment, influencing multiple signaling cascades. Work in transgenic animals indicates that overexpression of SOD1 impairs memory and synaptic plasticity [24]. Furthermore, treatment of hippocampal slices with catalase (CAT) restored synaptic plasticity [19]. One interpretation is that elevated SOD1 levels, particularly in older animals, can lead to excess hydrogen peroxide formation, altered redox signaling, and possibly neuronal damage [24]. In a recent study, viral vector mediated overexpression of SOD1 in hippocampal neurons was shown to reduce oxidative damage in young and middle-age rodents. Despite the decrease in oxidative damage, SOD1 expression impaired hippocampal-dependent cognition in middle-age rats, which was rescued by co-expression of CAT [30]. The results imply that increased SOD1 activity may increase production of hydrogen peroxide and result in redox-dependent changes observed during aging.

The current study was designed to determine whether overexpression of SOD1 in middle-age promotes an oxidized redox environment and associated changes in NMDAR function. The results confirm that overexpression of SOD1 reduced oxidative damage and impaired cognition in middle-age rats. In addition, we observed that SOD1 overexpression decreased glutathione peroxidase (GPx) activity and reduced the level of glutathione (GSH). Furthermore, SOD1 overexpression resulted in a decline in NMDAR-mediated synaptic responses and impaired LTP. Co-expression of CAT rescued the effects of overexpression of SOD1 on GSH levels, GPx activity, NMDAR function, and LTP. Finally, application of the reducing agent dithiothreitol (DTT) to hippocampal slices increased the NMDAR synaptic component in slices that overexpress SOD1, relative to slices that overexpress SOD1+CAT.

2. Materials and Methods

2.1. Animals

All treatments were approved by the Institutional Animal Care and Use Committee and were in accordance with guidelines established by the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals. Adeno-associated virus (AAV) containing antioxidant enzymes genes and green fluorescent protein (GFP) were stereotactically injected bilaterally into dorsal hippocampi of 17-month old male Fischer 344 rats (Harlan Laboratories). Rats were injected with SOD1+GFP (n = 11); or SOD1+CAT (n = 12); or GFP (n = 14) as a control. Behavioral testing in the water maze was initiated 1 month following virus injections.

2.2. AAV Viral Vector

Virus particles were produced and quantified by dot blot analysis, through the Powell Gene Therapy Center at the University of Florida (Gainesville, FL, USA). Plasmids for the self-complementary human SOD1 gene with a c-terminal myc tag (c-myc), human catalase, or GFP were cloned into pseudotyped rAAV2/5, containing the AAV2 terminal repeats and packaged in a rAAV5 capsid, which targets neurons in the brain [9,30]. Expression of each gene was driven by the chicken β -actin promoter and the cytomegalovirus-immediate early enhancer. Rats were anesthetized with ketamine/xylazine (90/10 mg/kg) and virus was stereotactically injected at 2 sites bilaterally in the hippocampus using glass pipettes. Each injection consisted of 2 μ L of GFP (dot blot vector genome titer (vg): 1.02×10^{13} vg/mL), 4 μ L 1:1 mix of SOD1 (dot blot titer: 1.14×10^{13} vg/mL) and GFP, or 3 μ L 2:1 mix of SOD1 and CAT (dot blot titer: 2.53×10^{13} vg/mL).

2.3. Behavior

Rats were first trained to find a visible platform using 15 trials separated in to 5 blocks. Three days later, rats were tested on a single-day spatial version of the water maze task, which is sensitive to the early emergence of memory deficits [17]. The spatial training protocol consisted of 15 trials separated into five blocks. A free swim probe trial with the platform removed (probe 1) was inserted between blocks 5 and 6. For the probe trial, the number of platform crossings was counted and the time spent in both goal and opposite quadrants was recorded and a discrimination index score was calculated as the (time spent in goal quadrant - time spent in opposite quadrant) / (total time in goal + opposite quadrant).

2.4. Biochemical assays

Starting one week after finishing behavioral testing, animals were euthanized using isoflurane (Webster, Sterling, MA, USA) and decapitated with a guillotine (Myneurolab, St Louis, MO, USA). For biochemical studies, the hippocampi were removed and flash frozen in liquid nitrogen. The samples were then stored at -80°C until further processing. Hippocampal tissues were ground into powder by mortar and pestle with liquid nitrogen. Half of the tissue powder was gathered in 1.5 mL tube and stored in -80°C for GSH measures and enzyme activity assays.

2.4.1. Measuring glutathione, glutathione peroxidase, and glutathione reductase—A glutathione (GSH) fluorescent detection kit (Arbor Assays LLC, Ann Arbor, MI, USA) was used to measure reduced/oxidized glutathione (GSH/GSSG) according to the manufactures instructions. Glutathione peroxidase (GPx) activity was measured by using a glutathione peroxidase assay kit (Cayman, Ann Arbor, MI, USA) and glutathione reductase (GR) activity was measured suing a GR fluorescent activity kit (Arbor Assays LLC, Ann Arbor, MI, USA).

2.4.2. Western blots—For western blots, tissues were homogenized in RIPA buffer supplemented with protease inhibitor, phosphatase inhibitor, and EDTA (Thermo scientific, Rockford, IL, USA), and incubated for 1 hour on ice with intermittent vortexing. The lysates were centrifuged at 20,000 g for 30 minutes at 4°C. Lysates were diluted 1/20 in a total of 50 µL of ddH₂O for measuring protein concentration on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 560 nm using a BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were diluted with lysis buffer to the same protein concentration. The samples were then mixed with 4X Laemmli's SDS-sample buffer [Tris-HCl (250 mM, pH 6.8), sodium dodecyl sulfate (8%), glycerol (40%), β-mercaptoethanol (8%) and bromophenol (0.02%)] (Boston BioProducts, Ashland, MA, USA) and heated to 100 °C for 5 minutes.

Samples (20 µg/lane) were loaded on 4-15 % gradient polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and run at 90 Volts in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) (Bio-Rad Laboratories, Hercules, CA, USA) until the blue indicator line reached the bottom of the gel. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare Biosciences, Pittsburgh, PA, USA) overnight at 50 Volts, 4°C in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol). Blots were then stained with Ponceau S and examined for transfer quality.

Blots were washed 5 min in Tris buffered saline with Tween 20 (TBST) (137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20) and subsequently blocked in 5% milk in TBST for 1 hour. Primary antibodies were then applied to blots overnight at 4 °C, washed 3 times (10 min each time) with TBST, and secondary antibodies applied for 1~2 hours at room temperature. Primary antibodies used for western blots included mouse anti CAT (1:700) (Abnova, Taipei City, Taiwan), mouse anti glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:6000) (EnCor Biotechnology, Gainesville, FL, USA), rabbit anti GFP (1:2000) (Life Technologies, Grand Island, NY, USA), mouse anti 4-hydroxy-2-nonenal (HNE) (1:100) (Abcam, Cambridge, MA, USA), mouse anti Myc-tag (1:1000) (Cell Signaling Technology, Danvers, MA, USA), and rabbit anti SOD1 (1:5000) (Abcam, Cambridge, MA, USA). Blots were again washed 4 times (10 min each time) with TBST and developed using Amersham ECL Plus Western Blot Detection Kit (GE Healthcare Biosciences, Pittsburgh, PA, USA) on BioMax film (Kodak). Blots were scanned using 6500 scanner (Bio-Rad, Hercules, CA USA), and densitometry determined using Image J.

Stripping of the membranes allowed us to re-probe with different antibodies, thus avoiding inconsistent sample loading between each electrophoresis blotting, and conserved the samples for other assays. Typically, the blot was first probed with antibody with lowest level of signal. The blot was then incubated in the stripping buffer (Thermo Scientific, Rockford, IL, USA) for 15 min at 37°C, and 3X washed with TBST, blocked with 5% milk in TBST and re-probed with another primary antibody made from different species. For example, if the first blot was probed with mouse antibody, then the second blot would be re-probed with antibody made from another species rather than mouse. This prevented using of the same secondary antibody in the first and second blotting, which may result to additional signals from the residual primary antibody after stripping. The blot was then stripped again and re-probed with either a third antibody or with anti-GAPDH antibody as loading control. Each membrane was stripped up to 3 times.

2.5. Immunofluorescence

Brains were postfixed in 4% paraformaldehyde followed by 30% sucrose in PBS (4°C). Sections (10-20 µm) were incubated with primary antibodies overnight at 4°C. Primary antibodies used for immunofluorescence included rabbit anti CAT (1:500) (Abcam, Cambridge, MA, USA), mouse anti Myc-tag (1:2000) (Cell Signaling Technology, Danvers,

MA, USA), and rabbit anti SOD1 (1:1000) (Abcam, Cambridge, MA, USA). The brain sections were then washed and incubated in corresponding Alexa 488 or 594 secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature. Sections were washed and counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) solution (0.1 $\mu\text{g/mL}$ in PBS) before mounting. Expression was confirmed using fluorescent microscopy (Zeiss Axioplan 2 upright fluorescent microscope, equipped with a QImaging Retiga 4000R Camera with RGB-HM-5 Color Filter and QImaging QCapture Pro 6.0 software; QImaging Surrey, BC Canada). To control for non-specific interactions of immunoglobulin molecules with the sample, a primary antibody control was used. The tissue was incubated with a non-immune immunoglobulin of the same isotype (for example, IgG_{2a} for c-myc control) and concentration as the primary antibody in 3% goat serum in PBS before incubation with secondary antibody, DAPI and mounting.

2.6. Electrophysiology

The methods for hippocampal slice preparation and recording have been published previously [5,29]. Briefly, the animals were deeply anaesthetized using isoflurane (Webster, Sterling, MA, USA), the brains were rapidly removed and hippocampi were dissected. Hippocampal slices (~400 μm) were cut parallel to the alvear fibers using a tissue chopper. The slices were incubated in a holding chamber at room temperature with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 2, KH₂PO₄ 1.25, MgSO₄ 2, CaCl₂ 2, NaHCO₃ 26, and D-glucose 10. At least 30 min before recording, slices were transferred to a standard interface recording chamber. The chamber was continuously perfused with oxygenated ACSF (95%-O₂ and 5%-CO₂) at the rate of 2 mL/min. The pH and temperature were maintained at 7.4 and $30 \pm 0.5^\circ\text{C}$, respectively. Humidified air (95% O₂, 5% CO₂) was continuously blown over the slices. Extracellular field excitatory postsynaptic potentials (fEPSPs) from stratum radiatum of the CA1 region of the hippocampus were recorded using glass micropipettes (4-6 M Ω) filled with recording medium (ACSF). Stimulating electrodes were localized to the middle of the stratum radiatum to stimulate CA3 inputs onto CA1. Biphasic stimulus pulses of 100 μs duration were delivered by a stimulator and alternated between the two pathways such that each pathway was activated at 0.033 Hz. The signals were amplified, filtered between 1 Hz and 1 kHz, and stored on a computer disk for off-line analysis. Two cursors were placed to cover the initial descending phase of the waveform and the maximum negative slope (mV/ms) of the fEPSP was determined by a computer algorithm which determined the maximum change across a set of 20 consecutively recorded points (20-kHz sampling frequency) between the two cursors.

For induction of LTP, the stimulation intensity was set to elicit 50% of the maximal fEPSP. After stable baseline recording at 0.033 Hz for at least 20 min, high frequency stimulation was delivered to the pathway at 100 Hz for 1 sec, repeated four times at 1 sec intervals using the baseline stimulus intensity and recorded for 60 min after delivery of LTP-inducing stimulation. A simultaneously recorded control pathway received the test stimulus but not the high frequency stimulation. The average fEPSP slope corresponding to the last 5 minutes from each pathway was used to compare changes in synaptic strength relative to the baseline. The NMDAR-mediated component of the field EPSP was obtained by incubating slices in ACSF containing low extracellular Mg²⁺ (0.5 mM), 6,7-dinitroquinoxaline-2,3-dione (30 μM), and picrotoxin (10 M). Input-output curves for the NMDAR response were constructed by measuring the slope of the EPSP for increasing stimulation intensities. To examine DTT effects, the NMDAR baseline synaptic response was set ~0.1 mV/msec and responses were collected for at least 10 min before and 60 min after drug application. The DTT dose (0.5 mM) was selected based on our previous studies [5,28].

2.7. Statistics

All statistical analyses were performed using StatView 5.0 (SAS Institute, Cary, NC, USA). Analyses of variance (ANOVAs) were used to establish main effects and interactions. Follow-up ANOVAs and/or Fisher's protected least significant difference post hoc comparisons with $p < 0.05$ were employed to determine specific differences. One group student *t*-tests were used to determine whether the discrimination index scores were different than that expected by chance and whether stimulation to induce LTP resulted in an increase in the synaptic response relative to baseline.

3. Results

3.1. Characterization of behavior on the water maze

Fig. 1 shows the mean escape path length across training blocks during cue and spatial discrimination training. A repeated measures ANOVA on path length for 5 blocks of cue discrimination training indicated an effect of training [$F(4,124) = 12.78$, $p < 0.0001$] and a treatment effect [$F(2,1124) = 4.54$, $p < 0.05$]. Post hoc analysis indicated that GFP rats required more training to acquire the cue discrimination strategy, such that the path length to find platform was greater than SOD1+GFP rats on blocks 2 and 3. Differences were not evident for the first block or the final two training blocks. Repeated measures ANOVAs within each group confirmed that all groups exhibited a decrease in path length during training. Thus, despite an initial difference on blocks 2-3, all groups were able to acquire the cue discrimination to the same extent (Fig. 1A).

Spatial discrimination was initiated 3 days later. Analysis of escape path length across the 6 training blocks indicated effects of training [$F(5,145) = 12.64$, $p < 0.0001$] in the absence of a treatment effect or an interaction. A probe trial between blocks 5 and 6 was employed to determine whether the animals acquired a spatial search strategy. SOD1+GFP rats exhibited the lowest mean discrimination index; however, an ANOVA failed to detect a treatment effect. In contrast, one-group *t*-tests comparing the discrimination index score to chance (i.e. score = 0) indicated that only GFP controls and the SOD1+CAT group acquired a spatial search strategy (Fig. 1B). Poor search behavior for SOD1+GFP animals was evident for platform crossings (Fig. 1C). An ANOVA for the number of platform crossings indicated a treatment effect [$F(2,31) = 3.5$, $p < 0.05$] and post hoc tests indicated that SOD1+GFP rats exhibited a decrease in platform crossings relative to the other two groups. The poor search behavior of SOD1+GFP animals was also evident in the time to make the first crossing of the center of the platform location [$F(2,31) = 4.66$, $p < 0.05$], such that the latency was increased for SOD1+GFP animals relative to the other two groups (Fig. 1D).

3.2. Characterization of enzyme expression and oxidative damage

Our previous work indicates that virus-mediated expression is largely limited to neurons of the hippocampus [30]. Injections of SOD1+GFP (Fig. 2A) and SOD1+CAT (Fig. 2B) resulted in co-localization within the soma and dendrites of hippocampal neurons. Western blots confirmed that GFP expression was only observed in rats injected with AAV to express GFP or SOD1+GFP and c-myc was only detected in rats injected with AAV to express myc tagged SOD1, including SOD1+GFP and SOD1+CAT (Fig. 3A). Two SOD1 bands were observed for the AAV-SOD1 injected rats (Fig. 3A) representing endogenous rat SOD1 (rSOD1), observed at 19 kDa, and a band representing the myc tagged human SOD1 was located at 23 kDa (Fig. 3A). For quantification, band intensities were normalized to GAPDH and compared for GFP ($n = 5-6$), SOD1+GFP ($n = 3-4$) and SOD1+CAT ($n = 4$) animals. Consistent with our previous work [30], expression of SOD1 was increased ~200% relative to GFP controls when the SOD1 vector is delivered in conjunction with the CAT vector. The total expression of SOD1 was significantly [$F(2,10) = 20.5$, $p < 0.0005$]

increased in animals injected with AAV-SOD1 and post hoc tests indicated a similar increase in total SOD1 in both SOD1+GFP and SOD1+CAT rats relative to GFP controls (Fig. 3B). Examination of the 19 kDa band indicated no difference (Fig. 3B) suggesting that viral mediated expression of SOD1 did not interfere with endogenous SOD1 levels. Our previous work indicates that injection of the CAT vector increases CAT expression by ~125-150%. In the current study, CAT expression was 159% of GFP animals. An ANOVA indicated a trend ($p = 0.1$) for a treatment effect and post hoc tests confirmed that CAT expression was increased in SOD1+CAT relative to the GFP only group. Finally, there was a treatment effect [$F(2,12) = 6.9$, $p < 0.05$] for the level of 4-hydroxy-2-nonenal (HNE), which is a commonly used marker of lipid peroxidation resulting from oxidative stress. Western blot analysis showed that the levels of HNE-modified proteins were greatest in the GFP control animals. Post hoc tests indicated significant reduction in the level of HNE-modified proteins in SOD1+CAT and SOD1+GFP relative to GFP controls (Fig. 3B). The results indicate that the overexpression of SOD1 through expression of SOD1+GFP or SOD1+CAT reduces lipid peroxidation, indexed by protein-bound HNE.

3.3. Effect of overexpression of SOD1 and CAT on glutathione

Despite a decrease in oxidative damage, overexpression of SOD1 can produce excess hydrogen peroxide, affecting redox sensitive signaling process that may be important for cognition. GSH and the oxidized form of GSH, glutathione disulfide (GSSG), represent the most abundant cellular redox couple and provide a major component for redox buffering [42]. To examine the redox environment, the level of GSH and GSSG were measured in the hippocampus of rats with overexpression of SOD1+GFP ($n = 11$), SOD1+CAT ($n = 9$), or GFP ($n = 12$) (Fig. 4). An ANOVA revealed a trend for a treatment effect for the free GSH levels ($p = 0.06$) with SOD1+GFP animals exhibiting the lowest GSH level. To measure total GSH, GSSG was reduced to GSH by adding extra glutathione reductase to the samples. An ANOVA indicated a treatment effect on total GSH [$F(2,29) = 10.14$, $p < 0.0005$] and post hoc comparisons indicated that overexpression of SOD1+GFP resulted in a >20% decrease in total GSH compared to GFP and SOD1+CAT treated rats. An ANOVA for the level of GSSG indicated a treatment effect [$F(2,29) = 4.96$, $p < 0.05$] and post hoc comparisons revealed that expression of SOD1+GFP resulted in a marked (~40%) decrease in GSSG compared to GFP and SOD1+CAT rats (Fig. 4). Together, the results indicate that overexpression of SOD1 alone results in a loss of GSH. Furthermore, the SOD1 associated loss in GSH is prevented by co-expression of CAT.

The activities of GPx and GR were measured in hippocampus of rats that overexpress SOD1+GFP ($n = 11$), SOD1+CAT ($n = 10$), or GFP ($n = 12$). An ANOVA on GPx activity revealed a significant treatment effect [$F(2,30) = 4.47$, $p < 0.05$]. Post hoc comparisons indicated that GPx activity of SOD1+GFP rats was reduced relative to SOD1+CAT (26%) and GFP (21%) rats (Fig. 5A). No treatment effect was observed for GR activity (Fig. 5B). The results indicate that the SOD1+GFP resulted in a loss of GSH and a decrease in GPx activity. Again, this change was reversed by co-expression of SOD1 with CAT.

3.4. NMDAR-mediated synaptic responses are reduced in rats with SOD1 overexpression

A decreased in NMDAR function and impaired induction of LTP has been linked to age-related changes in the intracellular redox environment in region CA1 [5,22,38,46]. If the loss of GSH and a decrease in GPx activity represents a more oxidized environment, then SOD1+GFP animals should exhibit impaired NMDAR function. To examine this possibility, synaptic responses were examined in hippocampal slices from SOD1+GFP ($n = 10$), SOD1+CAT ($n = 9$), and GFP ($n = 12$) animals. Examination of the input-output curves for the total synaptic response (SOD1+GFP: $n = 14$ slices; SOD1+CAT: $n = 14$ slices; GFP: $n = 21$ slices) indicated stimulation intensity effects for the presynaptic fiber volley (PFV)

[F(4,184) = 66.6, $p < 0.0001$] (Fig. 6A), total EPSP slope [F(4,184) = 49.02, $p < 0.0001$] (Fig. 6B), and the ratio of total EPSP slope/PFV [F(4,184) = 4.37, $p < 0.005$] (Fig. 6C). No group difference was observed for the PFV and the slope of the total EPSP. A repeated measures ANOVA on the input-output curve of the NMDAR synaptic response (SOD1+GFP: $n = 18$ slices; SOD1+CAT: $n = 17$; GFP: $n = 21$) indicated a significant effect of stimulation intensity [F(5,265) = 67.16, $p < 0.0001$] on the PFV, in the absence of a group effect (Fig. 6D). In contrast, an interaction between treatment and stimulation intensity was observed for the slope of the NMDAR EPSP [F(10,265) = 3.58, $p < 0.0005$] (Fig. 6E) and an effect of treatment was observed for the NMDAR EPSP slope/PFV [F(2,265) = 3.72, $p < 0.05$] (Fig. 6F). The treatment effect was due to decreased NMDAR responses in animals expressing SOD1+GFP. The results indicate that overexpression of SOD1+GFP has effects on synaptic transmission that are specific to NMDAR function. The decreased NMDAR response was reversed by co-expression of SOD1 with CAT.

The decrease in the NMDAR synaptic response observed during aging is associated with impaired induction of LTP [5,17]. Therefore, we determined whether LTP was impaired in slices from animals expressing SOD1+GFP (5 animals, 7 slices) relative to GFP (8 animals, 11 slices) or SOD1+CAT (7 animals, 9 slices). An ANOVA indicated a tendency ($p = 0.057$) for a treatment effect on the magnitude of LTP measured 60 min following delivery of high frequency stimulation and post hoc tests indicated that the magnitude of LTP was greater in GFP relative to SOD1+GFP. One group t -tests indicated that only slices from GFP and SOD1+CAT animals exhibited LTP, measured as an increase in the synaptic response above baseline (Fig. 7).

Finally, we examined the prediction that the decrease in NMDAR responses for SOD1 overexpression was due to an oxidized redox environment. For these studies, the stimulation intensity was adjusted to evoke an NMDAR-mediated synaptic response with a slope of ~ 0.1 mV/ms, and a baseline was recorded prior to application of the reducing agent DTT. Previous research indicates that the DTT mediated growth in the NMDAR-synaptic response is highly variable in middle-age animals and associated with cognitive function [28]. Therefore, the GFP animals (10 slices from 5 animals) were split according to their probe trial performance and one-tailed t -test [$t(8) = 2.37$, $p < 0.05$] confirmed that the DTT induced growth of the NMDAR response was greater ($213 \pm 41\%$, 5 slices) in slices obtained from two GFP rats in which the probe trial discrimination index scores were < 0 , relative to the DTT-mediated growth in the NMDAR synaptic response ($114 \pm 7\%$, 5 slices) for three GFP animals with discrimination index scores > 0.5 . Due to the variability, the responses for the GFP animals were not included in the analysis of DTT effects on SOD1+GFP (13 slices from 7 rats) and SOD1+CAT (13 slices from 8 rats) animals. Figure 8 shows the growth in the NMDAR-mediated synaptic response for the two groups and an ANOVA on the percent growth during the last 10 min of recording indicated that the magnitude of growth was significantly greater [F(1,24) = 9.39, $p < 0.01$] for slices expressing SOD1+GFP. The results provide the first evidence that increasing SOD1 or SOD1+CAT activity in middle-age influenced the redox regulation of NMDAR synaptic responses.

4. Discussion

Impairment in cognition and synaptic plasticity is observed in SOD1 transgenic mice or following viral-vector delivery of SOD1 to the hippocampus of rats [19,26,30], suggesting overexpression of SOD1 may provide a model for examining oxidative stress mechanisms in senescent neurophysiology. However, it is unclear if SOD1 overexpression involves an altered redox environment and a decrease in NMDAR function reported for aging animals [5,22,28,38,46]. In the current study, we specifically address the question of whether the

effects of SOD1 and CAT overexpression on cognition are associated with changes in the redox environment and redox regulation of NMDARs.

We confirmed that in middle-age animals, viral-vector mediated expression of SOD1 in hippocampal neurons reduced oxidative damage and impaired cognition [30]. The decrease in oxidative damage indicates that SOD1 overexpression increased the removal of superoxide. It is also possible that the loss of superoxide resulted in a reduced intracellular redox environment, which contributed to impaired cognition. Transgenic mice with an overexpression of extracellular SOD (i.e. SOD3) exhibit impaired memory and impaired LTP, which is thought to result from a decrease in activation of extracellular signal-related protein kinase and protein kinase C [45]. Extracellular signal-related protein kinase is activated by superoxide and inhibited by a reduced environment and excess GSH [2,13]. In this case, the loss of GSH and decrease in GSH redox cycle enzyme activity observed in the current study could be interpreted as a compensatory mechanism to maintain extracellular signal-related protein kinase signaling. However, several factors suggest that the effects of SOD1 overexpression in hippocampal neurons are different from those observed in extracellular SOD transgenic mice. First, SOD1 expression was associated with a decrease in NMDAR-mediated synaptic responses, which is consistent with a model of an oxidized environment observed during aging [5,22,28,38,46], and not observed in transgenic extracellular SOD mice [45]. Second, increasing CAT activity had no effect on LTP in extracellular SOD mice, indicating that impaired synaptic plasticity for extracellular SOD mice is not due to excess hydrogen peroxide [45]. In contrast, the current study demonstrates that elevated expression of CAT rescued cognition, GPx activity, GSH depletion, NMDAR synaptic responses, and promoted LTP, consistent with the idea that the effects of SOD1 overexpression were due to excess hydrogen peroxide. Finally, the ability of DTT to increase the NMDAR synaptic response was greater in the hippocampus of SOD1+GFP animals indicating a more oxidized environment. While the levels of superoxide and hydrogen peroxide were not measured, the results are consistent with the idea that overexpression of SOD1 resulted in a decrease in superoxide and an increase in hydrogen peroxide and support a dissociation between oxidative stress mediated oxidative damage and pro-oxidant redox environment in cognitive function and senescent physiology.

Impaired cognition associated with overexpression of SOD1 in hippocampal neurons is prominent in middle-age and not observed in young adult rats [30]. It may be important that middle-age is when cognitive deficits and redox modulation of NMDAR function first begin to emerge [28], suggesting that increased SOD1 activity may be interacting with the rise in oxidative stress during aging. Indeed, SOD1 overexpression can have positive or negative effects on neuronal survival and function depending on the cellular context [47]. Detrimental effects are observed under conditions in which excessive hydrogen peroxide accumulates due to increased production, decreased elimination, or a loss of GSH buffering [1,7,18,25]. In the current study, we observed a loss of GSH similar to that observed in the hippocampus over the course of aging [10,15,23,34,37,40,44]. Indeed, the loss of GSH in our middle-age rats mimics what is detected in the hippocampus of rats as they move from middle-age to advanced age [3]. GSSG and GSH levels are maintained by redox cycle enzymes, GPx and GR, and hydrogen peroxide irreversibly inactivates GPx [11,21,33,36,41]. Thus, an increase in hydrogen peroxide could underlie the observed decrease in the activity of GPx, which in turn contributes to loss of GSH and GSSG. In this regard, overexpression of CAT would provide an important pathway for preserving GPx activity and ultimately GSH levels. The marked loss of GSSH could result from the dethiolation of S-glutathionylated proteins [30] in order to maintain GSH levels and the redox environment [42]. Furthermore, GSSG is transported out of the cells during oxidative stress resulting in a loss of GSSG and total GSH [32]. While GSH:GSSG represent the most abundant cellular redox couple, assessment of the redox environment of neurons is complicated by the compartmentalization of

GSH:GSSG. Previous work demonstrates that GSH is mainly observed in astrocytes [27,43]. In contrast, neurons depend more on CAT rather than GSH to detoxify hydrogen peroxide [14]. However, hydrogen peroxide is membrane permeable such that surplus can be detoxified by GSH in glial cells. Together the results suggest that SOD1 overexpression in neurons may have influenced the redox environment of glial cells, possibly through excess hydrogen peroxide.

Oxidative stress and a pro-oxidant redox environment are thought to contribute to age-related cognitive decline and recent work suggests that redox modulation of NMDARs may underlie senescent synaptic function and the earliest signs of cognitive aging [17,28]. However, these studies are generally correlative in nature, describing redox-mediated changes during aging rather than directly modifying oxidative stress. The results of the current study indicate that SOD1 overexpression promoted an aging phenotype, impairing cognition, reducing the NMDAR-mediated component of synaptic transmission, and the ability to express LTP. Finally, DTT increased the NMDAR response to a greater extent in hippocampi that express SOD1+GFP relative to those that express SOD1+CAT, providing strong support to the hypothesis that increased SOD1 activity induces a more oxidized redox environment.

In summary, this is the first study to demonstrate that overexpression of SOD1 in neurons of middle-age animals promotes an oxidized redox environment. An oxidized redox environment during aging has been suggested to alter a number of Ca^{2+} signaling processes that are important for learning and memory [16,17]. Thus, it is likely that in addition to NMDAR function, overexpression of SOD1 influences other age-sensitive processes including Ca^{2+} -dependent regulation of cell excitability [6,31] and downstream signaling [5,8,12]. As such, overexpression of neuronal SOD1 may provide a model for studies on the role of oxidative stress in senescent physiology and the progression of age-related neurodegenerative diseases.

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List of Abbreviations

AAV	adeno-associated virus
ACSF	artificial cerebrospinal fluid
ANOVA	analyses of variance
c	myc- c-terminal myc tag
CAT	catalase
DTT	dithiolthreitol
DAPI	4'-6-Diamidino-2-phenylindole
fEPSP	field excitatory postsynaptic potential
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GPx	glutathione peroxidase

GR	Glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
HNE	4-hydroxy-2-nonenal
LTP	long-term potentiation
NMDAR	N-methyl-D-aspartate receptor
PFV	presynaptic fiber volley
rSOD1	rat superoxide dismutase 1
hSOD1	human superoxide dismutase 1
SOD1	superoxide dismutase 1
TBST	Tris buffered saline with Tween 20
vg	vector genome

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Fig. 1.

Effect of treatment on acquisition of cue and spatial discrimination on the water maze. (A) Mean distance to reach the escape platform for 5 blocks of cue and 6 blocks of spatial discrimination training. GFP expressing rats exhibited longer distance to reach the visible escape platform during block 2 and 3 of cue discrimination training. However, all groups (GFP *black circles*, $n=14$; SOD1+GFP rats: *gray circles*, $n=11$; SOD1+CAT rats: *open circles*, $n=12$) learned to reach the visible platform, as indicated by a significant overall decrease in distance across blocks. Spatial discrimination training was initiated 3 days later. The initial learning phase consisted of 6 blocks with an acquisition probe trial inserted between blocks 5 and 6 (arrow). Probe trial measures indicated impaired acquisition of a spatial search strategy in SOD1+GFP animals. (B) Mean+SEM discrimination index scores for GFP (black bars), SOD1+GFP (gray bars), and SOD1+CAT (open bars). Pound signs indicate a significant ($p < 0.05$) difference from chance (a score = 0) for SOD1+CAT and GFP animals. (C) Mean+SEM number of platform crossings and (D) latency for first platform crossing during the probe trial. Asterisk indicates a significant ($p < 0.05$) difference relative to SOD1+GFP.

Fig. 2. Co-localization of viral mediated overexpression of antioxidant enzymes in the hippocampus. (A) Merged images for cells in the hippocampus from a rat co-transduced with AAV-SOD1 (myc staining shown in red) and AAV-GFP (green) and (B) co-transduced with AAV-SOD1 (myc staining shown in red) and AAV-CAT (CAT staining shown in green). The merged figures indicate co-expression in neurons observed as yellow or orange. Calibration bars in (A) and (B) represent 100 μ m.

Fig. 3. Quantification of viral mediated overexpression of antioxidant enzymes in the hippocampus. (A) Western blots of hippocampal lysates from rats injected with viral vectors to express GFP, SOD1+GFP or SOD1+CAT. For SOD1, two bands were observed representing endogenous rat SOD1 (rSOD1 at 19 kDa) and the human myc tagged SOD1 (hSOD1 at 23 kDa). Antibodies against myc revealed a band at 23 kDa, only in animals injected with AAV-SOD1. GFP was only detected in rats injected with virus to express GFP or SOD1+GFP. Lipid peroxidation was visualized using an anti-4-hydroxy-2-nonenal (HNE) antibody. GAPDH was used as a loading control. (B) Quantification of western blot data of hippocampal lysates from rats injected with viral vectors to express GFP, SOD1+GFP, or SOD1+CAT. All densitometry was normalized by GAPDH and each bar represents the mean+SEM (n=3-6). Asterisk indicates a significant ($p < 0.05$) difference relative to GFP animals.

Fig. 4. Overexpression of SOD1 results in a loss of GSH, which is rescued by co-expression of SOD1+CAT. Bars represent the means+SEM. Asterisk indicates an increase relative to SOD1+GFP group.

Fig. 5. Overexpression of SOD1 results in a decrease in GPx, which is rescued by co-expression of SOD1+CAT. Bars represent the means+SEM for (A) GPx and (B) GR activity. Asterisk indicates an increase relative to SOD1+GFP group.

Fig. 6.

SOD1 overexpression has little effect on the total EPSP and decreases NMDAR synaptic responses. Input-output curves for (A) Presynaptic fiber volley (PFV) amplitude for the total synaptic response, (B) the total EPSP slope, (C) the total EPSP slope/PFV, (D) PFV amplitude for the NMDAR synaptic response, (E) the NMDAR EPSP slope, (F) the NMDAR EPSP slope/PFV for rats injected with GFP (filled circle), SOD1+GFP (gray circle), and SOD1+CAT (open circle). The numbers in the brackets indicate the number of slices/rats. The inserts in B and E illustrate total and NMDAR-mediated synaptic responses and the arrow indicate the PFV (open) and slope (filled) of the synaptic response.

Fig. 7.

SOD1 expression in the hippocampus of middle-age rats depresses LTP. (A) Time course of changes in the field EPSP obtained from hippocampal slices 10 min before and 60 min after LTP-inducing stimulation (arrow) to induce LTP for the rats expressing GFP (filled circle, $n = 11/8$, slices/animals), SOD1+GFP (gray circle, $n = 9/7$, slices/animals), SOD1+CAT (open circle, $n = 7/5$, slices/animals), and non-tetanized control path (line, $n = 27/20$, slices/animals). (B) Bar diagram showing the average magnitude of LTP during the last 5 min of recording [dotted area in (A)]. Asterisk indicates a significant ($p < 0.05$) difference from GFP. Pound signs indicate a significant difference from base line for GFP and SOD1+CAT animals.

Fig. 8.

Antioxidant enzymes contribute to redox regulation of NMDAR function. Time course of changes in the slope of NMDAR-fEPSP obtained from hippocampal slices 10 min before and 60 min after bath application of the reducing agent DTT (0.5 mM, solid line) for slices from SOD1+GFP (gray circle, $n = 13/7$, slices/animals) and SOD1+CAT (open circle, $n = 13/8$, slices/animals) animals.