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The hippocampus of Ames dwarf mice exhibits enhanced antioxidative defenses following kainic acid-induced oxidative stress

Sunita Sharma, Sharlene Rakoczy, Kristine Dahlheimer, and Holly Brown-Borg*

Department of Pharmacology, Physiology and Therapeutics, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58203, USA

Abstract

Introduction: The vulnerability of the hippocampus to the effects of aging has been found to be associated with a decline in growth hormone/insulin like growth factor-1 (GH/IGF-1), and an increase in oxidative stress. We have evidence that long-living GH-deficient Ames dwarf mice have enhanced antioxidant protection in the periphery but the protection in the central nervous system is less clear.

Material and methods: In the present study, we evaluated the antioxidative defense enzyme status in the hippocampus of Ames dwarf and wild type mice at 3, 12 and 24 months of age and examined the ability of each genotype to resist kainic acid-induced (KA) oxidative stress. An equiseizure concentration of KA was administered such that both genotypes responded with similar seizure scores and lipid peroxidation.

Results: We found that GH-sufficient wild type mice showed an increase in oxidative stress as indicated by the reduced ratio of glutathione: glutathione disulfide following KA injection while this ratio was maintained in GH-deficient Ames dwarf mice. In addition, glutathione peroxidase activity (GPx) as well as GPx1 mRNA expression was enhanced in KA-injected Ames dwarf mice but decreased in wild type mice. There was no induction of Nrf-2 (an oxidative stress-induced transcription factor) gene expression in Ames dwarf mice following KA further suggesting maintenance of antioxidant defense in GH-deficiency under oxidative stress conditions.

Discussion: Therefore, based on equiseizure administration of KA, Ames dwarf mice have an enhanced antioxidant defense capacity in the hippocampus similar to that observed in the periphery. This improved defense capability in the brain is likely due to increased GPx availability in Ames mice and may contribute to their enhanced longevity.

Keywords

Ames dwarf; Hippocampus; Oxidative stress; Glutathione; Glutathione peroxidase; Kainic acid

*Corresponding author. Tel.: +1 701 777 3949; fax: +1 701 777 4490. brownbrg@medicine.nodak.edu (H. Brown-Borg).

1. Introduction

Oxidative stress is a major factor implicated in aging (Ames et al., 1993; Stadtman and Berlett, 1997) and is also thought to be involved in the age-associated decline in learning and memory (Agarwal and Sohal, 1996; Dubey et al., 1996; Forster et al., 1996; Sohal et al., 1994). Studies suggest that age-related declines of cognitive and motor performance correlate with oxidative damage in specific brain regions (Forster et al., 1996) and that age-related impairment in spatial learning and memory may be alleviated by antioxidant treatment (D'Hooge and De Deyn, 2001).

The brain utilizes large amounts of oxygen, generates abundant free radicals and is relatively deficient in antioxidant enzymes (Cardozo-Pelaez et al., 2000; Halliwell, 1992a,b; Marklund et al., 1982) in comparison to other organ systems. In addition, the brain is rich in polyunsaturated fatty acids and is therefore more susceptible to damage induced by oxidative stress. Various studies have demonstrated that lipid peroxidation increases with aging (Joseph et al., 1996; Kasapoglu and Ozben, 2001; Leutner et al., 2001) while the activities of antioxidant enzymes decline with age and thus contribute to the aging process (Wickens, 2001). It has also been suggested that an age-related decline in circulating growth hormone and insulin like growth factor-1 (GH, IGF-1) may contribute to increased oxidative stress in the hippocampus with age (as evident by an increase in oxidized glutathione and concomitant decrease in the ratio of reduced glutathione/oxidized glutathione). Growth hormone replacement has been shown to attenuate this age-related increase in oxidative stress (Donahue et al., 2006).

Kainic acid (KA), an acidic pyrrolidine isolated from the seaweed *Digenea simplex*, is a cyclic analogue of the major excitatory neurotransmitter glutamate, and is an agonist for kainate receptors. It is used as a neurotoxic lesioning agent and specifically induces neuronal loss in the CA3 and CA1 regions of the hippocampus that have a high density of kainate receptors (Hikiji et al., 1993; Kamphuis et al., 1995; Schwob et al., 1980; Sun et al., 1992). Studies have reported that cell death induced by KA involves the generation of free radicals (Hirata and Cadet, 1997; Sun et al., 1992) and KA-induced seizure activity coincides with enhanced oxidative stress (Bruce and Baudry, 1995; Kim et al., 2000, 2002; Shin et al., 2008a). Kainic acid-induced neurotoxicity involves the peroxidation of lipids, a decrease in glutathione content and an accumulation of 4-hydroxynonenal, an especially neurotoxic end product of lipid peroxide decomposition (Farooqui et al., 2001). Furthermore, a variety of antioxidants (Duffy et al., 2008; MacGregor et al., 1996; Wang et al., 2004) and direct treatment with GSH have been shown to protect against KA-induced neurotoxicity (Ceccon et al., 2000; Saija et al., 1994).

Long-living mice are useful for assessing the relationship between oxidative stress and aging. The Ames dwarf mouse lives 50–70% longer than wild type mice due to a point mutation in the Prop 1 gene. This mutation impairs the normal development of the anterior pituitary resulting in non-detectable levels of circulating GH, thyroid stimulating hormone and prolactin. Ames dwarf mice are one-third the size of normal mice and the GH-deficiency results in a lack of peripheral IGF-1. Importantly, there is substantial evidence that Ames dwarf mice exhibit enhanced peripheral tissue antioxidant protection from aging-associated

oxidative stress as well as less vulnerability to oxidative and other stressors (Bokov et al., 2009; Brown-Borg, 2007; Brown-Borg et al., 1999, 2001, 2002; Brown-Borg and Rakoczy, 2000, 2003, 2005; Salmon et al., 2005). Centrally, we have shown that hippocampal tissues of Ames dwarf mice resist beta-amyloid-induced tau phosphorylation (a hallmark of Alzheimer's disease) when compared to wild type mice (Schrage et al., 2008). However, little is known about the hippocampal antioxidant status in vivo under conditions of oxidative stress in long-living mice.

We were therefore interested in determining the effect of KA on the antioxidant enzyme system in the dwarf hippocampus, an area integral for learning and memory in mammals. In a parallel study, we demonstrated that Ames dwarf mice exhibit enhanced learning compared to wild type mice and that they retain their hippocampal-based spatial memory after KA-induced hippocampal damage (Sharma et al., 2010). With this in mind, we hypothesized that this resistance to memory impairment could be partly due to the enhanced antioxidant defense capacity of Ames dwarf mice. Thus, in the present study, we document the effect of KA on copper-zinc superoxide dismutase (Cu-ZnSOD/SOD1), manganese superoxide dismutase (MnSOD/SOD2), glutathione (GSH), glutathione disulfide (GSSG), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR), and glutathione cysteine ligase (GCL) in the hippocampi of these animals and their wild type controls. The expression level of Nuclear factor-erythroid 2 p45-related factor-2 (Nrf-2), a major regulatory transcription factor for enzymes of glutathione metabolism, was also studied. Furthermore, lipid peroxidation as indicated by 4-hydroxy-2-nonenal (4-HNE) was demonstrated in the same area as the neuronal loss in the hippocampus of KA-injected animals. In addition, reactive astrogliosis in the hippocampus of these animals was observed following KA administration.

2. Material and methods

2.1. Animals

Ames dwarf mice were bred and maintained at the University of North Dakota vivarium facilities under controlled conditions of photoperiod (12 h light:12 h dark) and temperature (22 ± 1 °C) with ad libitum access to food (8640 Teklad 22/5 rodent diet with 22.6% crude protein, 5.2% fat, Harlan Laboratories) and water (standard laboratory conditions). The Ames dwarf (df/df) mice used in this study were derived from a closed colony with a heterogeneous background (over 25 years). Homozygous (df/df) or heterozygous (df/+) dwarf males were mated with carrier females (df/+) to generate dwarf mice. All procedures involving animals were reviewed and approved by the UND Institutional Animal Care and Use Committee. All of the wild type animals used in these studies were males to avoid sex steroid hormone effects while both male and female Ames dwarf mice were included since female dwarfs do not cycle and are thus not influenced by these hormones.

2.2. Kainic acid (KA) injections and tissue collection

Kainic acid (Ascent Scientific) was dissolved in normal saline as per manufacturer's instructions to make a stock solution. The same stock solution of kainic acid was used for all the animals injected with kainic acid to avoid batch to batch variation. Ames dwarf and wild

type mice ($n=6$ /group) at 3, 12 and 24 months of age were randomly divided to receive either normal saline (WT-SAL or DF-SAL) or KA at 15 mg/kg dose (WT-KA 15 or DF-KA 15). Additional groups of wild type mice at 3 and 12 months of age received 30 mg/kg of KA (WT-KA 30) or an equal volume of normal saline intraperitoneally.

Seizure intensity after KA injections was evaluated using a modified Racine rating system (Racine, 1972) as described previously (Kraft et al., 2006). Briefly, over a 2-h period following kainate injection, the seizure phenotype of the mice was quantified. Every 15 min, the maximal seizure characteristic observed was recorded as follows: 0, normal activity; 1, immobility/staring; 2, rigidity/tail extension/head bobbing; 3, repetitive movements, bilateral pawing, rearing, hind limb tremors; 4, minor seizure (severe forelimb tremor) or wobbling/jumping/falling; 5, Tonic clonic convulsions or multiple and/or prolonged occurrence of rating 4; 6, severe tonic-clonic seizure (observable loss of motor control); 7, death.

Mice were sacrificed 7 days following the KA challenge. Hippo-campi were collected from Ames dwarf and wild type mice, rapidly frozen and stored at -80°C until further analysis. A separate set of 3-month-old animals ($n=3$ /genotype/treatment group) was per-fused with paraformaldehyde 7 days after saline or KA injection for immunohistochemical analysis.

2.3. Antioxidant enzyme activity assays

Proteins were extracted from the tissues by homogenization on ice in buffer (CPE buffer -200 mM MOPS, 620 mM sucrose, 0.1 Mm EDTA, pH 7.2; Brown-Borg and Rakoczy, 2000) and the supernatant fraction was used for enzyme analysis. All enzyme activity levels were measured according to the previously described protocols. Protein quantification of the samples was performed using the Bradford assay (Bradford, 1976).

2.4. Estimation of GSH and GSSG

The ratio of GSH/GSSG is an indicator of oxidative stress: with a low ratio indicating increased oxidative stress with more GSH being transformed into GSSG than GSSG being converted to GSH. Determination of GSH was performed as previously described (Brown-Borg and Rakoczy, 2003) using the method of (Griffith, 1980). Each sample was assessed in triplicate, and the levels of GSH and GSSG were expressed as $\mu\text{mol/g}$ tissue. The GSSG (as $\text{GSH} \times 2$) was then subtracted from the total GSH to determine the actual level of GSH.

2.5. Estimation of GPx activity

The activity of GPx (EC 1.11.19) was determined according to a previously described method with some modifications (Flohe and Gunzler, 1984). The oxidation of NADPH by glutathione reductase (GR) was followed spectrophotometrically at 340 nm. Each sample was assayed in duplicate, and enzyme units were recorded as nmol of NADPH oxidized/min/mg protein using the extinction coefficient of 6.22×10^6 ml/mol.

2.6. Estimation of GR activity

Glutathione reductase (EC 1.6.4.2) activity was assayed spectrophotometrically by monitoring the oxidation of NADPH to NADP^+ by GR at 340 nm according to a previously described method (Carlberg, 1985) with some modification (Brown-Borg and Rakoczy,

2000). Samples were assayed in triplicate and GR activity was expressed as nanomoles of NADPH oxidized/minute/mg of protein (Wells and Deits, 1993).

2.7. Estimation of GST activity

The activity of GST (EC 2.5.1.18) was determined using previously described methods (Habig et al., 1974) (Mannervik et al., 1987). The formation of the conjugate of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) was monitored spectrophotometrically at 340 nm. Samples were assayed in duplicate and *S*-2,4-dinitrophenylglutathione produced was calculated from the linear slope of increasing absorbance using the extinction coefficient $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.8. Real time RT-PCR

Gene expression was evaluated in the hippocampus of 3, 12 and 24-month-old Ames dwarf and age-matched wild type mice using standard real-time RT-PCR techniques (Brown-Borg et al., 2009). Total RNA was extracted from tissues and equal amounts of RNA for the gene of interest and the reference gene, $\beta 2$ microglobulin ($\beta 2\text{M}$; Lupberger et al., 2002), was utilized to perform one-step real-time quantitative PCR using a QuantiTect SYBR Green RT-PCR kit (Qiagen). The gene specific primer pairs utilized are listed in Table 1. Gene expression was quantified using the comparative CT (threshold cycle) method (Heid et al., 1996). The amount of target DNA (in all the treatment groups) was normalized to the endogenous reference gene ($\beta 2\text{M}$) and compared relative to the control group (wild type mice saline group).

2.9. Western Blots of antioxidative enzymes

Proteins were extracted (one half of the hippocampus) in homogenizing buffer (CPE buffer; Brown-Borg and Rakoczy, 2000) and the supernatant fraction was used for analysis. Protein quantification of the samples was performed using a Bradford assay (Bradford, 1976). Protein samples were separated on 15% Criterion™ Precast Gels (Biorad) followed by transfer to PVDF membranes. Membranes were incubated overnight at 4 °C with antibodies to GCL (1:400, Thermoscientific), Cu-ZnSOD (1:1000, Calbiochem), MnSOD (1:100, Oxis), or GPx (1:100, Biogenesis) using standard immuno-blotting procedures and chemiluminescence. Quantification of protein levels was performed by densitometry using a UVP Biomaging system (Upland). Ponceau S was used as a loading control.

2.10. Immunohistochemistry

A separate group of 3-month-old mice ($n=3/\text{genotype/treatment}$) was perfused with phosphate buffered 4% paraformaldehyde pH 7.4, 7 days after saline or KA injection. Brains were removed and placed in 30% sucrose for cryoprotection. Coronal sections (20 μm thick) were obtained using a freezing sliding microtome (Leica 3000R) and collected into 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9% saline (phosphate-buffered saline). Sections were then preserved in a cryoprotectant solution consisting of 0.1 M phosphate buffer, pH 7.2 (50% v/v), sucrose (30% w/v), polyvinylpyrrolidone (1% w/v) and ethylene glycol (30% v/v) at -20°C until staining (Watson et al., 1986).

2.11. Fluoro-Jade C staining

Fluoro-Jade C (FJC) was used to stain for dying neurons in the brain using a previously described method (Schmued et al., 1997, 2005). Sections were rinsed, mounted, and air-dried. After pre-treatment with absolute alcohol followed by 70% ethanol, and distilled water, they were then oxidized in a solution of 0.06% KMnO₄. After rinsing, the sections were incubated for 20 minutes in a solution of 0.001% Fluoro-Jade C (Chemicon) containing 0.01% of DAPI (Molecular Probes, Invitrogen) in 0.1% acetic acid in the dark. The slides were rinsed, dried, cleared and coverslipped.

2.12. Staining for 4-HNE and glial fibrillary acidic protein (GFAP)

Free-floating sections from different mice were processed in parallel. For 4-HNE staining, tissue sections were rinsed followed by antigen retrieval in 10 mM citric acid buffer at 90–100 °C for 20 min. After extensive rinsing, sections were incubated in 0.1 M PBS containing 3% normal goat serum, 1% BSA, and 0.1% Triton-X 100 for 2 hr at room temperature. Sections were then incubated overnight at 4 °C with the rabbit anti-HNE (1:500, Calbiochem) followed by incubation with secondary biotinylated antibody (goat anti-rabbit, 1:500) for 1 h. Endogenous peroxidases were blocked by incubation in 3% H₂O₂ followed by incubation in streptavidin-horseradish peroxidase complex (Vector Labs, Burlingame, CA), according to the manufacturer's instructions (Vector Labs). Sections were developed in diaminobenzidine hydrogen peroxide, mounted onto slides, dried overnight, dehydrated, and coverslipped with Permount (Fischer Scientific). For GFAP staining, the procedure was similar except that antigen retrieval test with citric acid buffer was not performed and the primary antibody used was rabbit anti-GFAP (1:1000, DAKO).

For semi-quantification, FluoroJade C-stained degenerated neurons within the CA1 and CA3 subfields of hippocampus and GFAP-stained astrocytes within the CA1, CA3 and dentate gyrus, were counted from 20× digital images using Adobe Photoshop count tool (Adobe Photo-shop CS3 extended). Two areas (measuring 240 μm²) each from CA1 and CA3 subfields of hippocampus were used (3 sections/3 animals/genotype). The data were averaged for these two areas of CA1 and two areas of CA3 to obtain one measurement each for CA1 and CA3 subfields from each section. This average was then used for statistical analysis. The counting procedure used was similar to methods described in other reports (Liu et al., 2009; Mitruskova et al., 2005).

2.13. Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA, factors: genotype and treatment; Prism GraphPad). The treatment variable compared the treatment groups with the “equiseizure dose”: WT-KA 30 with DF-KA 15 at 3 and 12 months of age and WT-KA 15 mg/kg with DF-KA 15 mg/kg at 24 months of age. The Bonferroni's multiple comparisons test with a $p < 0.05$ adjusted significance level was used when appropriate to identify significant differences between groups. All data are presented as mean ± SEM.

3. Results

The present study examined the antioxidant enzyme status in the hippocampus of 3-, 12- and 24-month-old long-lived Ames dwarf mice as compared to the age-matched wild type siblings 7 days after the KA-induced oxidative stress. The body weights of the animals did not change over the course of the study in either genotype or saline control and kainic-acid treated groups after treatment. Body weights (g) of dwarf mice averaged 11.7 ± 0.2 pre-KA and 11.5 ± 0.2 post-KA at 3 months, 13.5 ± 1.0 pre-KA and 13.5 ± 1.1 post-KA at 12 months, and 15.6 ± 1.2 pre-KA and 15.6 ± 1.1 post-KA at 24 months. In wild type mice, the average body weights were 31.7 ± 1.7 pre-KA and 31.6 ± 1.3 post-KA at 3 months, 38.3 ± 2.1 pre-KA and 37.7 ± 2.5 post-KA at 12 months, 36.3 ± 1.6 pre-KA and 36.5 ± 1.1 post-KA at 24 months in the 15 mg/kg group. Similarly, the mean body weights of wild type mice in the 30 mg/kg group were 30.0 ± 0.9 pre-KA and 29.0 ± 0.6 at 3 months of age, and 38.7 ± 1.9 pre-KA and 35.4 ± 2.2 post-KA at 12 months of age.

3.1. Seizure activity following KA

In a preliminary study, we evaluated the sensitivity to seizures induced by various KA concentrations (ranging from 5–40 mg/kg) in wild type and age-matched Ames dwarf animals. Following KA injection, the mice were observed for two hours and were scored for seizure activity according to a modified Racine Scale (Kraft et al., 2006; Racine, 1972). Based on this early work, we found out that the total seizure scores were different ($p=0.0355$) at 3 months and appeared different ($p=0.1032$) at 12 months of age between wild type (15 mg/kg) and dwarf (15 mg/kg) mice but not at 24 months of age. However, there was no difference between the total seizure scores of Ames dwarf mice injected with 15 mg/kg KA and wild type mice with 30 mg/kg KA at 3 ($p=0.6399$) and 12 months of age ($p=1.000$; Fig. 1B). Therefore, to match the seizure scores, we selected 15 mg/kg dose of KA for dwarf and wild type mice and an additional group of 3- and 12-month-old wild type mice were also injected with 30 mg/kg kainic acid. The effects of KA on various parameters were compared between the “equiseizure” dose of 15 mg/kg in dwarf and 30 mg/kg in wild type for 3- and 12-month-old mice but at 15 mg/kg for both genotypes at 24 months of age. No seizure activity was observed in animals treated with an equal volume of normal saline (Fig. 1).

3.2. Cu-ZnSOD and Mn-SOD levels following KA-induced oxidative stress

3.2.1. Wild type mice—Three-month-old wild type mice receiving 30 mg/kg KA showed a tendency toward decreased Cu-ZnSOD protein levels. There was no difference in Cu-ZnSOD levels in 12- and 24-month-old wild type mice following kainic acid nor were differences detected in Mn-SOD levels at any age following KA.

3.2.2. Ames dwarf mice—Ames dwarf maintained similar protein levels of Cu-ZnSOD after KA injection. In contrast, Mn-SOD levels were decreased in Ames dwarf mice following KA at 12 months of age but the treatment x genotype interaction was significant. At 24 months of age, baseline Mn-SOD levels were significantly ($p<0.001$) higher in Ames dwarf mice as compared to wild type mice (Fig. 2); however, KA did not affect the level of this protein in the hippocampus.

3.3. GSH/GSSG ratio following KA-induced oxidative stress

3.3.1. Wild type mice—In our study, baseline GSH levels were increased significantly in wild type mice at 12 ($p<0.01$) and 24 ($p<0.05$) months of age as compared to younger animals. When the levels were compared between saline injected controls and KA injected mice within age group, an increase in GSH levels at 3 months ($p<0.05$ at 15 mg/kg KA; $p<0.01$ at 30 mg/kg KA) and at 12 months ($p<0.05$ at 30 mg/kg) of age was observed as compared to saline injected controls (Fig. 3A). There was also an increase in hippocampal GSSG levels in wild type animals after KA injection (30 mg/kg; $p<0.001$) at 3 and 12 months of age when compared to the saline injected group indicating increased oxidation of GSH (Fig. 3B). When the GSH/GSSG ratio was calculated, the ratio decreased by 65% and 75% at 3 and 12 months of age, respectively, in the 30 mg/kg KA-injected wild type mice ($p<0.001$). In addition, an increase ($p<0.05$) in the GSH/GSSG ratio at 3 months of age was observed in wild type mice treated with the lower dose of KA (15 mg/kg), possibly due to an increase in the GSH levels detected in these mice. There was no difference in the 24-month-old wild type mouse hippocampal levels of GSH, GSSG or the GSH/GSSG ratio following KA treatment.

3.3.2. Ames dwarf mice—In Ames dwarf mice, baseline GSH levels were increased at 12 months ($p<0.05$) as compared to mice at 3 months of age. Following KA injection, the hippocampal GSH levels did not change in Ames dwarf mice at any age. Similarly, there was no change in the GSSG levels in Ames dwarf mice following KA treatment (Fig. 3B). No differences in the GSH/GSSG ratios in dwarf mice were observed 7 days after administration of KA. Moreover, hippocampal GSH/GSSG ratios were elevated ($p<0.05$) in dwarf mice at 12 months of age as compared to 3-month-old mice. There were no differences in baseline (saline-injected group) GSH, GSSG, GSH/GSSG ratios between age-matched genotypes at 3, 12 or 24 months of age.

3.4. Glutathione peroxidase activity and gene expression levels following KA

As H_2O_2 accumulates, GPx, along with GSH as a substrate, converts the H_2O_2 to H_2O and GSSG. To determine if GPx gene expression in the hippocampus was similarly affected, we studied the expression of GPx1 and GPx4 mRNA (Fig. 4).

3.4.1. Wild type mice—In the present study, we observed a decrease in hippocampal GPx activity in 12-month-old wild type mice following treatment with 30 mg/kg KA as compared to saline-injected mice; however, there was a significant interaction in the analysis confounding the main effects (Fig. 4). Similar to the activity levels, GPx1 and GPx4 mRNA levels decreased following KA with a decrease at 12 and 24 months of age ($p<0.05$; Fig. 4B, C).

3.4.2. Ames dwarf mice—In contrast to wild type mice, no decrease in GPx activity was observed in the Ames dwarf mice despite the KA-induced oxidative stress and neuronal loss. In fact, 3-month-old dwarf mice appeared to have higher GPx activity following KA challenge (Fig. 4A). As found with activity levels, GPx1 and GPx4 mRNA expression levels in Ames mice were maintained with the exception of decrease in GPx4 mRNA following KA treatment at 12 months of age ($p<0.05$; Fig. 4C).

3.5. Glutathione-S-transferase and glutathione reductase activities following KA

Another family of cellular defense enzymes are the GSTs that also catalyze glutathione conjugation of 4-HNE (Esterbauer et al., 1991). The glutathione reductase enzyme maintains GSH in a reduced state thereby maintaining higher GSH/GSSG ratios.

3.5.1. Wild type mice—We did not observe any changes in hippocampal GST activity following KA treatment in wild type animals (WT-SAL: 49.35 ± 2.90 ; WT-KA 30: 45.13 ± 3.30 at 3 months; WT-SAL: 50.54 ± 1.70 ; WT-KA 30: 53.67 ± 3.70 at 12 months; WT-SAL: 48.70 ± 4.73 ; WT-KA 15: 46.05 ± 3.37 at 24 months). Despite the lower GSH/GSSG ratios in wild type mice, we did not observe any changes in the hippocampal GR activity following KA injection in these mice (WT-SAL: 6.97 ± 0.33 ; WT-KA 30: 7.16 ± 0.60 at 3 months; WT-SAL: 5.46 ± 1.39 ; WT-KA 30: 6.98 ± 0.34 at 12 months; WT-SAL: 3.01 ± 0.27 ; WT-KA 15: 2.73 ± 0.24 at 24 months). We also measured the mRNA expression levels of GR following KA treatment. In all age groups, the expression of GR tended to decrease following KA (data not shown).

3.5.2. Ames dwarf mice—Similar to wild type, Ames dwarf mice did not show any changes in GST activity following KA (DF-SAL: 41.02 ± 3.45 ; DF-KA 15: 39.11 ± 5.29 at 3 months; DF-SAL: 37.13 ± 1.47 ; DF-KA 15: 36.91 ± 2.32 at 12 months; DF-SAL: 45.49 ± 3.68 ; DF-KA 15: 41.03 ± 2.74 at 24 months). Furthermore, there were no changes in GR activity in Ames dwarf mice following this oxidative insult (DF-SAL: 3.66 ± 0.29 ; DF-KA 15: 3.78 ± 0.35 at 3 months; DF-SAL: 2.89 ± 0.15 ; DF-KA 15: 2.88 ± 0.14 at 12 months; DF-SAL: 2.76 ± 0.18 ; DF-KA 15: 2.30 ± 0.31 at 24 months). As in wild type mice, gene expression of GR tended to decrease following KA (data not shown) in Ames dwarf mice.

The activity of hippocampal GST was not influenced by age or genotype. While, in both genotypes the hippocampal GR activity decreased with age ($p < 0.05$).

3.6. Glutathione cysteine ligase levels following KA

Since GR activity was unchanged and yet GSH levels were maintained after KA in our study, we explored the *de novo* GSH synthetic pathway to see if synthesis was responsible for the replenishment of GSH. To determine if gene expression of the two subunits of the GCL enzyme (catalytic GCLc; modifier GCLm) were affected by KA, we also evaluated the mRNA expression levels of these two subunits in hippocampal tissue.

3.6.1. Wild type mice—Protein levels of GCL, the rate-limiting enzyme in the glutathione synthetic pathway tended to decrease in wild type mice following KA injection (Fig. 5A). Both GCLc and GCLm decreased ($p < 0.05$) following KA in wild type mice. At 3 and 24 months of age, GCLm was lower while at 12 months of age, the levels of GCLc were decreased significantly following KA injection ($p < 0.01$; Fig. 5B, C).

3.6.2. Ames dwarf mice—In contrast to wild type mice, GCL protein levels were maintained or were even higher in Ames dwarf mice following KA injection (Fig. 5A). Similarly, gene expression levels of both GCLc and GCLm did not change in Ames dwarf mice with the exception of a decrease at 12 months of age for GCLc ($p < 0.01$; Fig. 5B, C).

3.7. Nrf-2 expression levels following KA

Nrf-2 is a major regulator of several antioxidant enzymes including those of glutathione metabolism. Therefore, gene expression levels of Nrf-2 were evaluated.

3.7.1. Wild type mice—Nrf-2 expression decreased in 12-month-old mice wild type animals following 30 mg/kg KA injection ($p<0.05$). In old, wild type mice (24 months of age), we observed an increase in Nrf-2 expression indicating a response to oxidative stress (Fig. 6).

3.7.2. Ames dwarf mice—Intriguingly, Nrf-2 expression also decreased in the Ames dwarf at 3 and 12 months ($p<0.05$) in the KA-injected group as compared to saline-injected controls, suggesting that these animals do not need Nrf-2 support to maintain their antioxidant enzyme system. In contrast to wild type mice at 24 months of age, Nrf-2 expression levels in Ames dwarf mice did not change. In addition, at 24 months of age, Ames dwarf mice had a higher baseline expression of Nrf-2 as compared to age-matched wild type mice ($p<0.05$; Fig. 6).

3.8. Kainic acid-induced lipid peroxidation in the CA1 and CA3 subfields of the hippocampus

4-Hydroxy-2-nonenal (4-HNE) was used as a marker of lipid peroxidation. To provide support for the hypothesis that lipid peroxidation is occurring in the same areas as the neuronal loss, parallel sections were stained with FJC.

3.8.1. Wild type mice—We examined 4-HNE immunoreactivity in the hippocampus of 3-month-old KA-injected mice, as compared to control saline injected animals. A representative micrograph of 4-HNE staining in the hippocampal CA3 and CA1 subfields is shown in Fig. 7. Strong 4-HNE immunoreactivity was detected in the CA3 and CA1 (Fig. 7C, M) subfields of wild type mice at 30 mg/kg KA. There was very little immunoreactivity to 4-HNE in wild type mice injected with saline or KA at 15 mg/kg. The control sections that were incubated with normal goat serum (instead of primary antibody) showed no staining. Parallel sections stained with FJC indicated that a significant loss of hippocampal pyramidal cells occurred in the same CA3 and CA1 subfields (Fig. 7H, R; area with the highest density of kainate receptors) in wild type (30 mg/kg) mice.

3.8.2. Ames dwarf mice—Similar to wild type mice, 4-HNE immunoreactivity was detected in the CA3 and CA1 subfields of the hippocampus of Ames dwarf mice injected with 15 mg/kg KA (Fig. 7E, O). Parallel sections stained with FJC showed neurodegeneration in the same CA3 and CA1 subfields in Ames dwarf mice injected with 15 mg/kg KA (Fig. 7J, T). The hippocampus obtained from saline injected animals did not show any immunoreactivity to 4-HNE or FJC.

There were no differences in the number of FJC positive cells between the wild type mice injected with 30 mg/kg KA and Ames dwarf mice injected with 15 mg/kg KA in CA3 ($p=0.4053$) and CA1 ($p=0.9776$) subfields as reported in the previous study (Sharma et al., 2010).

3.9. Reactive astrogliosis following KA

3.9.1. Wild type mice—In saline-injected wild type animals, a basal level of immunostaining of GFAP was observed in the hippocampus (Fig. 8A, F, K). Evaluation of GFAP by immunohistochemistry revealed the presence of KA-induced reactive astrocytes in the hippocampus of wild type mice. There was no increase over that of baseline staining in wild type mice at the dose of 15 mg/kg KA (Fig. 8B, G, L). However, the “equiseizure” KA dose of 30 mg/kg in wild type produced an increase in immunoreactivity as compared to saline-injected controls (Fig. 8C, H, M).

3.9.2. Ames dwarf mice—In saline-injected Ames dwarf mice (Fig. 8D, I, N), a basal level immunostaining of GFAP was observed in the hippocampus. However, the “equiseizure” KA dose of 15 mg/kg in Ames dwarf mice produced an increase in immunoreactivity as compared to saline-injected controls (Fig. 8E, J, O).

The quantification of GFAP in three different areas of hippocampus (CA1, CA3 and dentate gyrus) also showed that there was an increase in GFAP positive cells following the “equiseizure” dose of KA in wild type (30 mg/kg) and in Ames dwarf (15 mg/kg) mice in CA1 (Fig. 8P; wild type $p<0.001$; Ames dwarf $p<0.05$), CA3 (Fig. 8Q; Ames dwarf $p<0.05$), and the dentate gyrus (Fig. 8R; wild type $p<0.05$; Ames dwarf $p<0.05$). In addition, when genotypes were compared, Ames dwarf had greater numbers of GFAP positive cells in CA1 (Fig. 8P; $p<0.05$) as compared to wild type mice injected with KA (30 mg/kg). This difference was not significant in the CA3 subfield and the dentate gyrus.

4. Discussion

The current study documents the differences in the cellular antioxidant defense enzymes in the hippocampus of animals with different life spans. In addition, this study shows that long living Ames dwarf mice exhibit enhanced antioxidant defense following KA-induced oxidative stress as compared to wild type mice.

Various studies have reported that KA-induced seizure activity coincides with enhanced oxidative stress in the limbic system (Bruce and Baudry, 1995; Hirata and Cadet, 1997; Kim et al., 2000, 2002; Shin et al., 2008a). Consistently, it has been reported that KA-induced seizure activity impairs glutathione homeostasis and negatively correlates with the GSH/GSSG ratio or GPx activity (Shin et al., 2008b). Thus, one objective of our study was to examine the effects of a CNS targeted oxidative insult on the oxidative defense system in long living mice. In preliminary studies we evaluated different concentrations of KA for the ability to produce similar seizure activity and oxidative stress between genotypes. We found that Ames dwarfs were more sensitive to KA on weight basis. The genotype differences observed could be due to the fact that the percentage of body fat is lower in Ames mice than in the corresponding normal controls (Heiman et al., 2003), a factor known to alter drug pharmacokinetics. Kainic acid is also known to cause damage to the blood brain barrier (Reynolds and Morton, 1998; Zucker et al., 1983). Thus, an alternative explanation for the increased susceptibility of Ames dwarf mice to KA could be that more damage to the blood–brain barrier occurred in dwarf mice as compared to wild type mice. Considering the differences in seizure activity and neuronal damage produced by different doses of KA, we

chose the 15 mg/kg KA in the Ames dwarf mice that matched the 30 mg/kg KA dose in 3- and 12-month-old and 15 mg/kg in 24-month-old wild type mice for seizure score and oxidative insult. Therefore, this study utilizes the “equiseizure dose” between wild type and Ames dwarf mice to compare various parameters.

The Cu-ZnSOD protein, the first line of defense in eliminating superoxide radicals, showed a tendency to decrease in wild type mice at 3 months of age, but levels were maintained in Ames dwarf mice following KA. Our findings agree with a report showing that kainate-induced hippocampal DNA damage is attenuated in Cu-ZnSOD transgenic mice (Hirata and Cadet, 1997). It has been demonstrated that cytoplasmic Cu-ZnSOD alterations were more pronounced than that of mitochondrial MnSOD in the vulnerable hippocampal CA1 subfield of the brain with aging (Hayakawa et al., 2008). Similarly, we did not find changes in MnSOD levels following KA in both genotypes except in dwarf mice at 12 months of age, but the results were confounded in this group. In our study we found higher levels of baseline MnSOD in Ames dwarf mice as compared to wild type mice at 24 months of age indicating better defense against superoxide radicals well into old age in Ames dwarf mice.

Apart from SOD, glutathione is an important endogenous antioxidant in the brain. It has been shown to protect against the toxic effects of lipid peroxidation products such as HNE (Cooper and Kristal, 1997) and thus protects the cell from glutamate and *N*-methyl-D-aspartate (NMDA) excitotoxicity (Levy et al., 1991; Miyamoto et al., 2008). During this process, GSH itself is oxidized to form GSSG. A higher GSH/GSSG ratio thus reflects a more efficient glutathione system and a lower ratio is considered an indication of oxidative stress (Joshi et al., 2007; Schafer and Buettner, 2001). An imbalance in the levels of GSH/GSSG could be detrimental to cell survival. Kainic acid produces a decrease in the cellular content of glutathione in a dose- and time-dependent manner (Oyama et al., 1997; Shin et al., 2009). In addition, the decrease in GSH is associated with increased levels of GSSG and therefore, a lower ratio of GSH/GSSG is usually evident after KA (Shin et al., 2008a).

Similar to these previously reported studies, we also found an increase in GSSG and a decrease in the GSH/GSSG ratio following KA in wild type mice. A low GSH/GSSG ratio could promote an increased free radical load and oxidative stress (Bains and Shaw, 1997). It was surprising that we did not observe the expected decline in GSH following KA injection. There is a possibility that the glutathione was replenished by 7 days following KA administration. A temporal study might show the depletion of glutathione in the initial few days following KA injection. Nevertheless, the maintenance of the GSH/GSSG ratio in the Ames dwarf mice indicates preservation of this cellular redox system following KA-induced neuronal loss. A plausible explanation of maintenance of this antioxidant activity despite the neuronal loss could be due to increased levels of GSH in the surviving cells in the region.

Recent studies have also demonstrated that oxidative stress-induced alterations in the GSH/GSSG ratio in favor of GSSG can trigger apoptosis, independent of free radicals and GSH levels, suggesting that GSSG alone is capable of inducing apoptosis (Pias and Aw, 2002a,b). An increase in GSSG was found in the hippocampus of wild type mice in our study suggesting that these mice may have a compromised capacity for defense against oxidative stress and are thus, more vulnerable to neuronal cell death. Oxidative stress also negatively

affects synaptic plasticity and cognitive function through the oxidation of synaptic proteins (Scheff et al., 2005; Wu et al., 2006). We have, in fact, observed an impairment in spatial memory in wild type mice after KA-injection, while dwarf mice maintain their spatial memory at pre-KA levels (Sharma et al., 2010).

Glutathione peroxidase, which converts GSH to GSSG, is especially important in protecting the brain against lipid peroxides (Benzi and Moretti, 1995). In a traumatic brain injury model it was reported that GPx protects against oxidative stress and reduced GPx activity was associated with elevated levels of 4-HNE and acrolein (Ansari et al., 2008). In this study, we expected GPx activity to decrease with KA-induced oxidative stress. As predicted, this was the case for wild type mice suggesting utilization of GPx by wild type animals, which is in accordance with the finding that GSSG levels were increased in these mice. However, the GPx activities were not decreased in Ames dwarf mice. This maintenance of GPx activity in response to KA-induced oxidative stress further supports the evidence that Ames dwarf mice have an enhanced capacity or reserve to counteract free radicals in the CNS as has been shown in peripheral tissues. An upregulation of GPx has been suggested to be responsible for the reduction in early oxidative stress and long term sparing of neurons, thus rescuing the deficits in hippocampal-dependent spatial memory following traumatic brain injury (Tsuru-Aoyagi et al., 2009). We believe that the increased GPx activity in the hippocampus observed in our study is a similar adaptive defense mechanism against KA-induced free radical damage in Ames dwarf mice. In contrast, the decrease in mRNA expression for both GPx1 and GPx4 in wild type mice correlates with the decreased activity in GPx enzymes in these mice. GPx1 and GPx4 mRNA expression levels were not altered in Ames dwarf mice following KA, except GPx4 at 12 months of age. It is difficult to explain this exception based on the data as a whole, thus we propose to do more work on various subtypes of GPx at different age groups and different time points following KA injection to draw final conclusions.

We subsequently assessed expression levels of Nrf-2, a basic leucine-zipper transcription factor that regulates enzymes of glutathione metabolism and protect cells from oxidative stress (Kensler et al., 2007). In conditions of oxidative stress, Nrf-2 dissociates from Keap 1, its cytosolic inhibitor, and translocates to the nucleus, where it binds to antioxidant-response elements (AREs). This binding leads to the transcriptional induction of genes involved in glutathione metabolism (Kensler et al., 2007). Nrf-2 is critical for maintaining the GSH redox state via transcriptional regulation of GR and protecting cells against oxidative stress (Harvey et al., 2009). Kainic acid treatment has been shown to induce Nrf-2 in an attempt to maintain redox homeostasis (Kraft et al., 2006; Ogita et al., 2004). Short-term treatment with KA leads to the inhibition of glycogen synthase kinase-3 β (GSK-3 β) and correlates with the translocation of Nrf-2 to nucleus (Rojo et al., 2008). However, a more prolonged KA treatment can lead to GSK-3 β activation and reduced amounts of nuclear Nrf-2 levels resulting in down-regulation of Nrf-2-regulated antioxidant genes. We speculate that the “equiseizure” dose of KA in wild type mice at 12 months of age produces a prolonged oxidative stress resulting in a down-regulation of Nrf-2 and thus limiting the antioxidant capacity of the hippocampus in these mice. A similar situation may occur in the Ames dwarf mice at 12 months of age, but considering the findings of the GSH/GSSG ratio and GPx activity in these mice, we propose that Ames dwarf mice probably did not need the induction

of Nrf-2 because their constitutive enzyme system is capable of counteracting the KA-induced oxidative stress. Guo and Mattson (2000) also demonstrated that 2-deoxy-D-glucose administration generates a mild metabolic stress, leading to an increased resistance of neurons to subsequent, more severe insults, including excitotoxic, ischemic and oxidative injury. We believe that Ames dwarf mice could be under a similar mild oxidative stress due to their GH/IGF-1 status and therefore exhibit an upregulated antioxidant enzyme system. The evidence that the baseline Nrf-2 expression is high in Ames dwarf mice at older ages (24 months) as compared to wild type mice further supports the hypothesis that Ames dwarf mice have enhanced antioxidant defense mechanisms in the hippocampus. Furthermore, a recent study described that KA induces more prolonged and fatal seizures in Nrf-2 null mice than in wild type mice (Kraft et al., 2006). This evidence lends support to our data showing that the low levels of Nrf-2 in wild type mice at 24 months of age result in higher seizure activity at lower doses of KA (15 mg/kg).

Our data in the hippocampus showed significant HNE immuno-reactivity in KA-injected animals, strongly suggesting the involvement of oxidative stress. 4-HNE is an α,β -unsaturated aldehyde and is one of the major products of oxidation of membrane lipid polyunsaturated fatty acids (Esterbauer et al., 1991). Accumulation of 4-HNE is considered to play a role in the aging process (Hayakawa et al., 2008) and detection of 4-HNE is considered to be one of the most reliable indices of lipid peroxidation (Waeg et al., 1996). The formation of 4-HNE after KA injection is a time-dependent phenomenon observed in degenerating CA1 and CA3 fields of the hippocampus and precedes KA-induced cell death (Ong et al., 2000a). The areas of 4-HNE staining observed in our study (7 days post-KA) overlapped with the same areas of the hippocampus where degenerating neurons were detected, in accordance with previously reported studies (Chung and Han, 2003; Ong et al., 2000a). Although this study did not directly assess oxidative stress and cell loss in adult (12 months) and aging (24 months) animals experiencing a similar intensity of behavioral seizures, the degree of oxidative stress (as evident by 4-HNE staining) produced by 15 mg/kg in dwarf mice was similar to wild type mice injected with 30 mg/kg KA at 3 months of age. Based on these observations and a previous study, we can speculate that at a similar intensity of seizures, oxidative stress and neuronal loss will be similar in different age groups (Liang et al., 2007).

We have thus demonstrated that KA is causing HNE production leading to neuronal loss in hippocampus. It was also important to assess the reaction of glial tissue in the hippocampus following KA. We used GFAP staining, a protein that has been shown to be a reliable indicator of astrocytic activation and is increased in the hippocampus in response to KA injection (Eng et al., 2000). In addition, GFAP immunoreactivity reaches a peak 7 days following KA injections (Benkovic et al., 2004), identical to the time point used in our study. Furthermore, GPx and GFAP have been shown to co-localize in reactive astrocytes, suggesting that GPx is specifically induced in this particular type of glial cell in response to neuronal injury (Shin et al., 2008b). Glial overactivity causes the release of trophic factors and is thought to create a favorable environment for neuronal sprouting (Shetty et al., 2004, 2005). In our study we also found an increase in the GFAP positive astrocytes following KA in wild type mice (30 mg/kg) and Ames dwarf (15 mg/kg) mice. This finding substantiates two of our previous observations. One, that the equiseizure doses used in this study are

comparable. And two, that Ames dwarf mice have more reactive astrocytes (CA1 subfields; as obtained by semiquantification of GFAP staining) that may contribute to the observed genotype differences in GPx activities following KA.

It has also been demonstrated that even though GSH decreases in neurons after KA injection, there is an upregulation of GSH synthesis in reactive astrocytes 3 days to 6 weeks after kainate injection (Ong et al., 2000b). This might explain the maintenance of glutathione levels in Ames dwarf mice 7 days after KA injections. These data are also supported by the fact that in Ames dwarf mice the mRNA expression of GCLc and GCLm and protein levels of GCL were maintained following KA. We speculate that either the hippocampal cells of Ames dwarf are less severely damaged by the KA or the surviving cells exhibit enhanced antioxidative capacity.

Another cellular defense enzyme that is also abundant in astrocytes is GST (Cooper and Kristal, 1997). Increased expression of GST protects neuronal cells against oxidative stress induced by HNE (Lovell et al., 1998). It is unclear why hippocampal GST and GR activity remained unchanged after KA injection in both genotypes in our study. A possible explanation for the lack of a response to KA could be that these enzymes are replenished after an initial decrease, a hypothesis that can only be confirmed by examining the hippocampus at different time points after KA administration.

Thus, the results obtained in this study show that Ames dwarf mice are more vulnerable to KA-induced seizures, but that they exhibit enhanced antioxidant defense against this oxidative stress. It is plausible that the lack of GH and IGF-1 in Ames dwarf mice is responsible for increased seizure susceptibility yet is also responsible for triggering enhanced antioxidant defenses and increased stress resistance. We have previously shown that the hippocampus of the Ames mouse is more resistant to B-amyloid toxicity (Schrag et al., 2008).

In summary, this study demonstrates that at an equiseizure dose of KA, Ames dwarf mice exhibit enhanced antioxidant defense following KA-induced oxidative stress in the hippocampus as shown by higher GSH/GSSG ratios and GPx activities especially in the younger age groups. Furthermore, Nrf-2 is not induced in Ames dwarf mice during an oxidative challenge and yet these animals maintain adequate levels of antioxidant enzymes in the hippocampus to combat oxidative stress. This could be partially due to reactive astrogliosis, which in turn increases the levels of GPx. There is also a possibility that after KA-induced neuronal loss, the remaining viable cells in the hippocampus of Ames dwarf mice maintain a better reserve of antioxidant capacity as compared to wild type mice. Further studies are needed to explore other mechanisms responsible for the enhanced defense against oxidative stress in Ames dwarf mice.

Note: Following the submission of this manuscript, a report was published in *Endocrinology* (Gine et al., 2010) showing that rats with developmental hypothyroidism are extremely sensitive to KA-induced seizures due to increased expression of kainate receptors in the hippocampus. We are currently evaluating the levels of GluR5, GluR6, GluR7, KAR1 and

KAR2 to determine if an increase in these kainate receptors underlies the heightened sensitivity to KA in Ames dwarf mice.

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Fig. 1.

Mean (A) and total (B) seizure scores. Seizure score was assessed for 2 h following KA injection. The treatment groups at different ages included wild type mice receiving 15 mg/kg KA (WT-KA 15), wild type mice receiving 30 mg/kg KA (WT-KA 30), and Ames dwarf mice receiving 15 mg/kg KA (DF-KA 15). WT-KA 15 is represented by solid line with open circles, WT-KA 30 is represented by solid line with closed triangles, and DF-KA 15 is represented by dashed line with solid squares (A). The table indicates the mean total seizure scores following KA in 3-, 12- and 24-month-old wild type mice and Ames dwarf mice (B). Data are presented as mean±SEM. * indicates a significant difference (* p <0.05) between WT-KA 15 and DF-KA 15. In all age groups and both genotypes $n=6$ for each treatment group.

Fig. 2.

Cu-Zn SOD and Mn SOD protein levels in the hippocampus of wild type and Ames dwarf mice. The levels of Cu-Zn SOD (2A) and Mn SOD (2B) were assessed in the hippocampi of 3-, 12- and 24-month-old mice in both genotypes in saline and KA-injected animals. Data are presented as mean $\pm$ SEM. * indicates a significant difference ($**p<0.01$) between pre- and post KA data and # indicates (### $p<0.001$) baseline difference between wild type and Ames dwarf mice. In all age groups for both genotypes $n=5-6$ for each treatment group.

Fig. 3.

The levels of reduced glutathione (GSH), oxidized glutathione (GSSG), and the GSH/GSSG ratios in the hippocampus of wild type and Ames dwarf mice. The levels of GSH (3A) and GSSG (3B) were assessed in the hippocampi of 3-, 12- and 24-month-old mice in both genotypes in saline and KA-injected animals. The GSH/GSSG ratio (3C) was calculated as shown. Data are presented as mean $\pm$ SEM. * indicates a significant difference (** $p < 0.01$) between pre- and post KA data. In all age groups for both genotypes $n=5-6$ for each treatment group.

Fig. 4.

Glutathione peroxidase (GPx) activity and mRNA expression levels in the hippocampus of wild type and Ames dwarf mice. The activity of GPx (4A) was assessed in the hippocampi of 3-, 12- and 24-month-old mice in both genotypes after saline and KA injection. The mRNA expression levels of GPx1 (4B) and GPx4 (4C) were assessed in the hippocampi of 3-, 12- and 24-month-old mice in both genotypes after saline and KA injection. Data are presented as mean±SEM. * indicates a significant difference ($p<0.05$) between pre- and post-KA data. In all age groups for both genotypes $n=5-6$ for each treatment group.

Fig. 5.

Glutamyl cysteine ligase (GCL) protein levels and mRNA expression in the hippocampus of wild type and Ames dwarf mice. The protein levels of GCL (5A) were assessed in the hippocampi of 3-, 12- and 24-month-old mice in both genotypes after saline and KA injection. Glutamyl cysteine ligase catalytic (GCLc; 5B) and modulatory (GCLm; 5C) subunit mRNA expression levels were assessed in the hippocampi of 3-, 12- and 24-month-old mice in both genotypes after saline and KA injection. Data are presented as mean ± SEM. Indicates a significant difference ($p < 0.05$, ** $p < 0.01$) between pre- and post-KA data. In all age groups for both genotypes $n = 5-6$ for each treatment group.

Fig. 6.

Nuclear erythroid factor related factor-2 (Nrf-2) mRNA expression levels in the hippocampus of wild type and Ames dwarf mice. KA-induced changes in the levels of Nrf-2 expression were assessed in wild type and Ames dwarf mice. Data are presented as mean $\pm$ SEM. * indicates a significant difference ($p < 0.05$) between pre- and post KA data. In all age groups for both genotypes $n=5-6$ for each treatment group.

Fig. 7.

Representative micrograph of 4-hydroxy-2-nonenal (4-HNE) staining in the CA1 and CA3 subfields of hippocampi obtained from wild type and Ames dwarf mice 7 days after KA administration. Panels A–E and K–O show 4-HNE staining in CA3 and CA1 subfields respectively. Panels F–J and P–T show FJC staining from similar CA3 and CA1 subfields in different treatment groups in both wild type and Ames dwarf mice. In both genotypes $n=3$ for each treatment group. Scale bars represent 50 μ m in all images.

Fig. 8.

Representative micrographs of GFAP immunostaining in the hippocampus of wild type and Ames dwarf mice at 3 months of age. Different treatment groups with GFAP positive astrocytes are shown (A–O). Micrographs A–E show the whole hippocampus and dentate gyrus from micrographs F–O. Scale bars from micrographs A–E represent 200 μm , F–J represent 100 μm and K–O represent 50 μm . The quantification of GFAP positive cells is as shown in the bottom panel (P–R). Data are presented as mean $\pm$ SEM. * indicates a significant difference (** $p<0.01$, *** $p<0.001$) between pre- and post-KA data and # indicates (## $p<0.01$) difference between KA-injected wild type and Ames dwarf mice. In both genotypes $n=3$ for each treatment group.

Table 1

Primer pairs utilized for gene specific real time RT-PCR.

Gene	GeneBank accession no.	Primer 5'- 3'
β2M	NM_00975	For - AAG TAT ACT CAC GCC ACC CA Rev - AAG ACC AGT CCT TG
IGF-1	NM_010512	For - CTG AGC TGG TGG ATG CTC TT Rev - CAC TCA TCC ACA ATG CCT GT
IGF-1R	NM_010513	For - ACT GAC CTC ATG CGC ATG TGC TGG Rev - CTC GTT CTT GCC CCC GTT CAT
GPX1	NM_008160	For - TCA GTT CGG ACA CCA GGA GAA Rev - CTC ACC ATT CAC TTC GCA CTT C
GPX4	NM_008162	For - GCA TCC CGC GAT GAT TG Rev - TCG ATG TCC TT GGC TGA GAAT
Gclc	NM_010295	For - GGA GGC GAT GTT CTT GAG AC Rev - CAG AGG GTC GGA TGG TTG
Gclm	NM_008129	For - GAC TCA CAA TGA CCC GAA AGA Rev - GAT GCT TTC TTG AAG AGC TTC CT
Nrf2		For - CTC GCT GGA AAA AGA AGT G Rev - CCG TCC AGG AGT TCA GAG G