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The influence of gender, age and treatment time on brain oxidative stress and memory impairment induced by D-galactose in mice

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HIGHLIGHTS

- Age and treatment time but not gender affect D-gal-induced brain ROS generation.
- Gender, age and treatment time affect D-gal-induced brain oxidative stress.
- Gender, age and treatment time affect D-gal-induced spatial memory deficits.

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ABSTRACT

Chronic exposure to D-galactose (D-gal) serves as a model for age-related oxidative damage and cognitive dysfunction. However, methods used, including the dose and treatment time of D-gal as well as the gender, age and strain of animals used, vary greatly among published articles. In this study, we investigate the effect of gender, age and treatment time on brain oxidative stress and spatial memory deficits induced by D-gal in mice, respectively. Eight-week-old female mice injected with 100 mg/kg D-gal per day, for 6 weeks, did not show spatial memory impairment or high levels of hydroxyl radical, protein carbonyl and malondialdehyde in brain homogenates, although brain reactive oxygen species were increased when compared with saline control mice. In contrast, both 8-week-old male mice and 24-week-old female mice receiving 100 mg/kg D-gal for 6 weeks, or 8-week-old female mice receiving 100 mg/kg D-gal for 10 weeks showed spatial memory deficits and significant increases in the above oxidative markers, compared with their corresponding controls. These results demonstrate that D-gal-induced brain oxidative stress and spatial memory impairment are dependent upon exposure time of D-gal, plus gender and age of the animals used. The findings can serve as a useful guide for successfully establishing D-gal induced age-related oxidative damage models.

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1. Introduction

Oxidative stress is a consequence of an imbalance between pro-oxidant and antioxidant defenses, causing accumulation of reactive oxygen species (ROS). The accumulated ROS lead to oxidative modification of biomolecules such as lipids, proteins and nucleic acids, in turn impairing cellular function and causing cellular senescence [1]. The brain is particularly vulnerable to oxidative damage in part because of its relatively high rate of oxidative metabolic activity, low antioxidant defenses, and high levels of unsaturated fatty acids that are substrates for peroxidation reactions [2]. Therefore, protection against oxidative stress is critical for delaying brain aging and preventing neurodegenerative disorders, such as Alzheimer's disease. In this regard, it would be important to establish suitable animal models for assessing the safety and therapeutic efficacy of brain antioxidants.

D-Galactose (D-gal) is a reducing sugar that can generate ROS during its metabolism in vivo [3]. Rats and mice chronically treated with D-gal show brain oxidative stress and cognitive dysfunction, accompanied with several hallmarks of age-related neurodegeneration such as cholinergic degeneration [4], impairment of synaptic plasticity and neurogenesis [5], altered expression of amyloid-beta metabolism-associated molecules [6], reactive gliosis [7] and neuroinflammation [8]. Therefore, chronic injection of D-gal serves as an animal model for age-related brain oxidative damage and anti-aging pharmacology research.

According to the Pubmed Database of National Library of Medicine, more than 500 papers have been published using D-gal

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model. However, the dose (50–1250 mg/kg) [3,9] and treatment time (14–90 days) [6,10] as well as the animal's gender (female or male) [11,12], age (1–15 months) [13,14] and strain (Kunming, ICR, C57BL/6 J, Swiss albino, etc) [6,10–12] vary in the current literature (for detailed information see electronic Supplementary material). Each of these factors may affect model establishment and therapeutic efficacy of antioxidants. Aside from the dosing effect [15], the influence of other aforementioned factors on D-gal-induced brain oxidative damage remains unclear.

In this study, we investigate the effects of age, gender and treatment time on establishing a mouse D-gal induced aging model. Our results demonstrate that the each factor affects D-gal-induced brain oxidative stress and cognitive impairment.

2. Materials and methods

2.1. Animals and experimental design

C57BL/6 strain mice were housed at 20–25 °C, 60% relative humidity, 12/12-h light/dark cycle, with food and water available ad libitum. Eighty mice were divided into eight groups, with each group ($n = 10$) receiving one of the following treatments (Table 1; for detailed information of animal groups see electronic Supplementary material).

2.2. Y-maze test

On the 43rd day (Group 1–6) or 71st day (Group 7–8), mice were tested for spatial learning and memory using the Y-maze which is designed to examine the innate tendency of mice to explore novelty [12]. The number of arm entries, percentage of entry per arm, and total distance travelled were calculated (For the detailed protocols see electronic Supplementary material).

2.3. Brain tissue homogenate preparation

Immediately after Y-maze test, mice were terminated by decapitation. The hippocampus was promptly dissected and homogenized in ice-cold Locke's buffer to obtain a tissue concentration of 10 mg/ml. The homogenate (10%) was centrifuged at 12,000 × *g* at 4 °C for 10 min and the supernatant was used for biochemical analysis.

2.4. Measurement of ROS in brain homogenates

ROS was measured based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) to 2′,7′-dichlorofluorescein (DCF) as described previously [16].

2.5. Measurement of oxidative parameters in brain homogenates

Commercial kits (Nanjing Jiancheng Bioengineering Institute, China) were used to detect hydroxyl radical (HR), protein carbonyl (PC) and malondialdehyde (MDA) levels in brain homogenates. Detailed methods have been described in our previously published report [7].

2.6. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Differences among means were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. $P < 0.05$ was defined as significant.

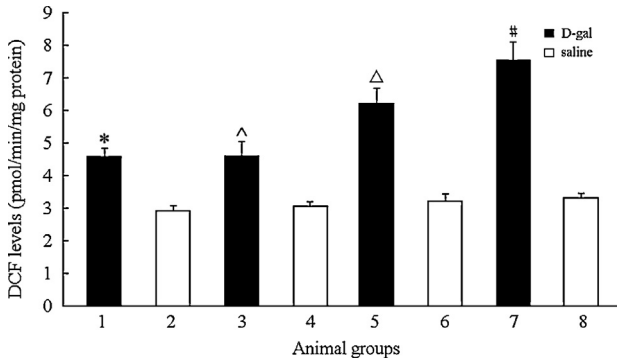

Fig. 1. Analysis of brain ROS levels. Data represent means ± SEM. from six mice per group. * $P < 0.05$, vs. Group 2 and Group 4; ^ $P < 0.05$, vs. Group 2 and Group 4; △ $P < 0.05$, vs. Group 1, Group 2 and Group 6; # $P < 0.05$, vs. Group 1, Group 2 and Group 8.

3. Results

3.1. The effect of gender, age and treatment time on ROS levels in the brain of mice injected D-gal

We first determined the effect of gender on D-gal-induced brain ROS production by comparing levels of DCF fluorescence in the hippocampal homogenate of mice from Group 1, Group 2, Group 3 and Group 4. Two-way ANOVA revealed a significant effect of treatment ($F_{1,20} = 32.497$, $P < 0.001$). Tukey's post hoc tests confirmed that Group 1 and Group 3 had high DCF fluorescence levels compared with Group 2 and Group 4, respectively (all $P < 0.01$; Fig. 1). No effects were observed for gender and treatment-gender interaction ($F_{1,20} = 0.083$, $P = 0.777$; $F_{1,20} = 0.034$, $P = 0.855$, respectively).

We then investigated the effect of age on D-gal-induced brain ROS production by comparing levels of DCF fluorescence among Group 1, Group 2, Group 5 and Group 6. Two-way ANOVA revealed significant effects of treatment, age and treatment-age interaction ($F_{1,20} = 60.081$, $P < 0.001$; $F_{1,20} = 10.676$, $P = 0.004$; $F_{1,20} = 5.026$, $P = 0.036$, respectively). Group 5 exhibited the highest levels of DCF fluorescence ($P < 0.001$, vs Group 1; $P < 0.01$, vs Group 1; $P < 0.001$, vs Group 6, respectively; Fig. 1). Group 1 also had higher DCF fluorescence levels than Group 2 and Group 6 ($P < 0.01$; $P < 0.05$, respectively). There was no difference between Group 2 and Group 6 ($P > 0.05$).

Finally, we examined the effect of exposure time on D-gal-induced brain ROS production among Group 1, Group 2, Group 7 and Group 8. Two-way ANOVA revealed significant effects of treatment, time and treatment-time interaction ($F_{1,20} = 79.786$, $P < 0.001$; $F_{1,20} = 26.094$, $P < 0.001$; $F_{1,20} = 15.158$, $P = 0.001$, respectively). Group 7 had highest DCF fluorescence levels ($P < 0.001$, vs Group 1, Group 2 or Group 8; Fig. 1). There was no difference between Group 8 and Group 1 or Group 2 (both $P > 0.05$).

3.2. The effect of gender, age and treatment time on D-gal-induced brain oxidative stress

We further addressed whether D-gal-induced ROS production resulted in brain oxidative stress, and could be affected by gender, age or treatment time. The gender effect was investigated by comparing levels of HR, PC and MDA levels, markers of oxidative damage in DNA, proteins and lipids in the hippocampal homogenate of mice from Group 1, Group 2, Group 3 and Group 4. Two-way ANOVA revealed significant effects of treatment, gender and treatment-gender interaction on the levels of HR ($F_{1,20} = 5.802$, $P = 0.026$; $F_{1,20} = 50.735$, $P < 0.001$; $F_{1,20} = 14.402$, $P = 0.048$, respectively), PC ($F_{1,20} = 10.435$, $P = 0.004$;

Table 1
Experimental design.

Animal group	1	2	3	4	5	6	7	8
Gender	Female	Female	Male	Male	Female	Female	Female	Female
Age (wks.)	8	8	8	8	24	24	8	8
Exposure time (wks.)	6	6	6	6	6	6	10	10
Treatment agents	D-gal	Saline	D-gal	Saline	D-gal	Saline	D-gal	Saline

$F_{1,20}$ = 25.767, P < 0.001; $F_{1,20}$ = 8.414, P = 0.009, respectively) and MDA ($F_{1,20}$ = 13.76, P = 0.001; $F_{1,20}$ = 26.493, P < 0.001; $F_{1,20}$ = 12.51, P = 0.002, respectively). Group 3 showed significantly higher levels of HR, PC and MDA than Group 1 (all P < 0.05), Group 2 (all P < 0.001) and Group 4 (all P < 0.001) (Fig. 2A–C). There was no significant difference among the other three groups.

The effect of age on D-gal-induced brain oxidative stress was examined by comparing levels of HR, PC and MDA among Group 1, Group 2, Group 5 and Group 6. The treatment, age and treatment-age interaction had significant effects on the levels of HR ($F_{1,20}$ = 8.261, P = 0.009; $F_{1,20}$ = 25.085, P < 0.001; $F_{1,20}$ = 5.56, P = 0.029, respectively), PC ($F_{1,20}$ = 17.613, P < 0.001; $F_{1,20}$ = 16.499, P = 0.001; $F_{1,20}$ = 8.7, P = 0.008, respectively) and MDA ($F_{1,20}$ = 40.726, P < 0.001; $F_{1,20}$ = 49.588, P < 0.001; $F_{1,20}$ = 30.266, P < 0.001, respectively). Group 5 showed significantly higher levels of HR, PC and MDA than Group 1 (all P < 0.01), Group 2 (all P < 0.001) and Group 6 (all P < 0.001) (Fig. 2A–C). There was no significant difference among the other three groups.

The effect of exposure time on D-gal-induced brain oxidative stress was determined by comparing levels of HR, PC and MDA among Group 1, Group 2, Group 7 and Group 8. Significant effects of treatment, time and treatment-time interaction on the levels of HR ($F_{1,20}$ = 14.169, P = 0.001; $F_{1,20}$ = 34.19, P < 0.001; $F_{1,20}$ = 12.645, P = 0.002, respectively), PC ($F_{1,20}$ = 48.27, P < 0.001; $F_{1,20}$ = 50.153, P = 0.001; $F_{1,20}$ = 35.692, P < 0.001, respectively) and MDA ($F_{1,20}$ = 35.422, P < 0.001; $F_{1,20}$ = 38.066, P < 0.001; $F_{1,20}$ = 28.061, P < 0.001, respectively) were observed. Group 7 showed significantly higher levels of HR, PC and MDA than Group 1 (all P < 0.001), Group 2 (all P < 0.001) and Group 8 (all P < 0.001) (Fig. 2A–C). There was no significant difference among the other three groups.

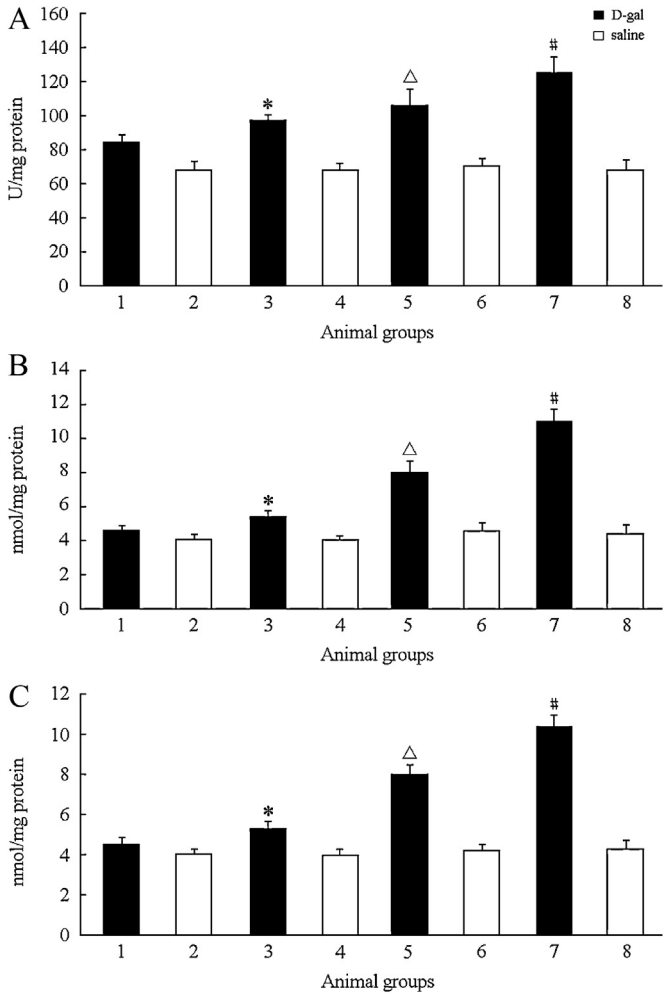

Fig. 2. Analysis of brain oxidative parameters. (A) Levels of hydroxyl radial (HR). (B) Levels of protein carbonyl (PC). (C) Levels of malondialdehyde (MDA). Data represent means ± SEM. from 6 mice per group. * P < 0.05, vs. Group 2 and Group 4; △ P < 0.05, vs. Group 1, Group 2 and Group 6; # P < 0.05, vs. Group 1, Group 2 and Group 8.

3.3. The effect of gender, age and treatment time on D-gal-induced spatial memory deficits

The effect of gender on D-gal-induced spatial memory deficits was examined by comparing Y-maze memory performance among Group 1, Group 2, Group 3 and Group 4. Significant effects of treatment, gender and treatment-gender interaction on the number of the novel arm entries ($F_{1,36}$ = 4.964, P = 0.033; $F_{1,36}$ = 7.493, P = 0.01; $F_{1,36}$ = 12.907, P = 0.001, respectively). Group 3 showed lower number of the novel arm entries than Group 1 (P < 0.01), Group 2 (P < 0.001) and Group 4 (P < 0.01). There was no significant difference among the other three groups. For the percentage of time spent in the novel arm, significant effects of treatment ($F_{1,36}$ = 11.33, P = 0.002), but not gender ($F_{1,36}$ = 3.12, P = 0.086) or treatment-gender interaction ($F_{1,36}$ = 2.317, P = 0.137), were observed. Group 3, but not Group 1, showed decreases in the percentage of time spent in the novel arm, compared with Group 2 or Group 4 (both P < 0.01; Fig. 3A and B). There was no difference between Group 1 and Group 3 (P > 0.05) or between Group 2 and Group 4 (P > 0.05). In addition, effects of treatment and gender on the total distance traveled were not significant ($F_{1,36}$ = 0.211, P = 0.649; $F_{1,36}$ = 0.157, P = 0.694, respectively), indicating locomotor activities were not different among the groups.

The effect of age on D-gal-induced spatial memory deficits was examined by comparing Y-maze data among Group 1, Group 2, Group 5 and Group 6. Significant effects of treatment, age and treatment-age interaction on the number of the novel arm entries ($F_{1,36}$ = 4.324, P = 0.045; $F_{1,36}$ = 16.61, P < 0.009; $F_{1,36}$ = 11.629, P = 0.002, respectively) and the percentage of time spent in the novel arm ($F_{1,36}$ = 16.302, P < 0.001; $F_{1,36}$ = 12.801, P = 0.001, $F_{1,36}$ = 6.756, P = 0.013, respectively) were observed. Group 5 had lower numbers of the novel arm entries and a lower percentage of time spent in the novel arm than Group 1 (P < 0.01, P < 0.001, respectively), Group 2 (both P < 0.001) or Group 6 (P < 0.01, P < 0.001, respectively) (Fig. 3A and B). There was no significant effect of treatment or gender on the total distance traveled ($F_{1,36}$ = 0.777, P = 0.384; $F_{1,36}$ = 3.979, P = 0.054, respectively).

The effect of exposure time on D-gal-induced spatial memory deficits was also examined by comparing Y-maze data among Group 1, Group 2, Group 7 and Group 8. Significant effects of treatment, time and treatment-time interaction on the number of the

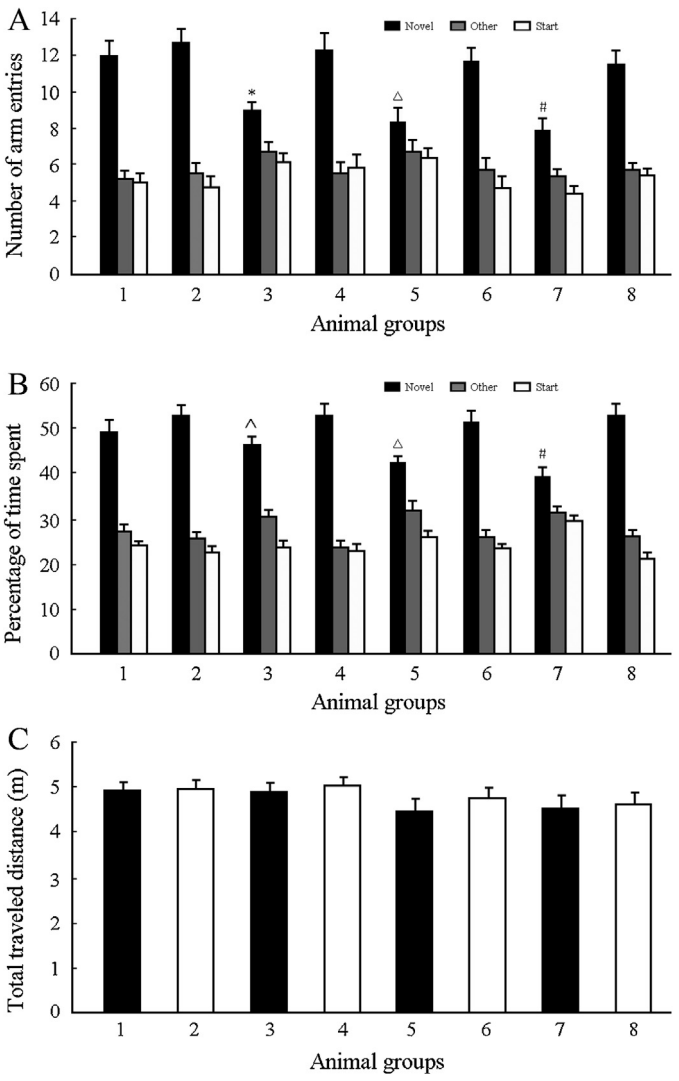

Fig. 3. Evaluation of spatial memory. (A) The number of arm entries, (B) The percentage of time spent in each arm. (C) The total distance travelled. Data represent means ± SEM. from 10 mice per group. * $P < 0.05$, vs. Group 1, Group 2 and Group 4; ^ $P < 0.05$, vs. Group 2 and Group 4; △ $P < 0.05$, vs. Group 1, Group 2 and Group 6; # $P < 0.05$, vs. Group 1, Group 2 and Group 8.

novel arm entries ($F_{1,36} = 4.528$, $P = 0.05$; $F_{1,36} = 18.789$, $P < 0.001$; $F_{1,36} = 12.907$, $P = 0.001$, respectively) and percentage of time spent in the novel arm ($F_{1,36} = 45.265$, $P < 0.001$; $F_{1,36} = 24.26$, $P < 0.001$; $F_{1,36} = 25.743$, $P < 0.001$, respectively) were observed. Group 7 had lower numbers of the novel arm entries and a lower percentage of time spent in the novel arm than Group1 (both $P < 0.001$), Group 2 (both $P < 0.001$) or Group 8 ($P < 0.01$, $P < 0.001$, respectively) (Fig. 3A and B). No significant effect of treatment or gender on the total distance traveled was observed ($F_{1,36} = 0.318$, $P = 0.576$; $F_{1,36} = 1.577$, $P = 0.847$, respectively).

4. Discussion

Chronic exposure to D-gal injection has been employed as a rapid, economic and stable aging model, which is widely used for brain aging or anti-aging pharmacology research. However, the methods vary greatly among the published articles. In the present study, we have demonstrated that D-gal-induced brain oxidative stress and spatial memory impairment are dependent on the gender and age of the animals used, as well as on the exposure time of D-gal.

Gender differences are widespread in a variety of both normal and pathological brain functions [17]. In agreement with this notion, the present results have shown that 8 week-old male mice, but not female mice, display mild spatial memory impairment and brain oxidative stress after exposure to 100 mg/kg D-gal per day for 6 weeks. This finding suggests that female adolescent mice are more resistant than male adolescent mice to oxidative stress induced by D-gal. This conclusion is also supported by previous studies from two independent research groups [11,12]. Eight-week-old female C57BL/6J mice i.p. injected daily with 100 mg/kg D-gal for 6 weeks had no changes in spatial memory revealed by Y-maze test [12]. Conversely, 2–3 month male Swiss albino mice that received 100 mg/kg of D-gal administered subcutaneously for 6 weeks had impaired memory performance in Morris water maze task and an elevated plus maze paradigm [11]. We considered that endogenous ovarian hormones may contribute to the resistance of female adolescent mice to oxidative damage, since there are numerous evidences that endogenous ovarian hormones, especially estrogen, have distinctive antioxidative abilities [18]. A previous study from our laboratory showed that ovarian hormone depletion by ovariectomy exacerbates memory impairment and forebrain cholinergic degeneration in D-gal treated rats, which can be reversed by 17-β estradiol replacement [19]. Taken together, these results suggested that, compared with female adolescent mice, male adolescent mice may be more suitable for use in D-gal-induced age-related neurobehavioral outcomes.

Age-related oxidative stress is a well known phenomenon, although the exact mechanism is not fully understood [1]. The present results show that female mice receiving 100 mg/kg D-gal for 6 weeks, at 24 week-old, but not 8 week-old, exhibited higher levels of ROS, HR, PC and MDA in the brain and increased memory impairment than the two age control groups. These data suggest that the adult female brain is more sensitive to D-gal-induced oxidative damage than the adolescent female brain. One possibility of age-dependent oxidative stress induced by D-gal may be associated with a decrease in antioxidative function with age [20]. The evidence for this presumption is that a daily injection of D-gal (500 mg/kg body weight) for 7 weeks significantly reduces the expression of endogenous antioxidant enzyme genes, including Cat, Gpx1, Sod1 and Sod2 in the liver of 22-week-old ICR mice, but only Cat gene expression in 6-week-old mice [21]. These findings suggest that adult female mice may be available for obtaining a better efficiency of D-gal-induced oxidative stress, compared with adolescent female mice.

The present study also revealed that 8 week-old female mice receiving 100 mg/kg D-gal per day for 10 weeks, but not 6 weeks, displayed brain oxidative stress and memory deficits. These data suggest that D-gal-induced brain aging is a time-dependent process. In addition, increased ROS levels were observed in the hippocampus of 8 weeks-old female mice with a 6-week treatment of D-gal, but significant oxidative damage in DNA, proteins and lipids did not occur. This indicates that accumulation of ROS precedes oxidative damage in the brain during the aging process. Our previous study revealed that astrocytes were mildly activated without neuron impairment and synapse loss in the hippocampus of mice receiving intraperitoneal injection of D-gal (200 mg/kg per day) for 2 weeks [10]. Mild reactive astrogliosis is shown to protect against brain oxidative stress via glutathione production [22]. Thus, activated astrocytes may be responsible for maintaining oxidative homeostasis in the early stages of this aging model.

In conclusion, we have provided evidence that brain oxidative stress and spatial memory deficits induced by the chronic administration of D-gal is greatly dependent on the gender and age of mice used, as well as on the treatment time. These findings may help explain the variable effectiveness in D-gal mimicking brain senescence in the current literature. The finding, together with the

dose-dependent effect of D-gal on behavioral impairment reported previously, can serve as a useful guide for successfully establishing a D-gal aging mouse model.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.04.038.

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