Aquaporin-4 deficiency exacerbates brain oxidative damage and memory deficits induced by long-term ovarian hormone deprivation and D-galactose injection

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Abstract

Astrocyte dysfunction is implicated in pathogenesis of certain neurological disorders including Alzheimer’s disease (AD). A growing body of evidence indicates that water channel aquaporin-4 (AQP4) is a potential molecular target for the regulation astrocyte function. Recently, we reported that AQP4 expression was increased in the hippocampus of an AD mouse model established by long-term ovarian hormone deprivation combined with D-galactose (D-gal) exposure. However, pathophysiological roles and mechanisms of AQP4 up-regulation remain unclear. To address this issue, age-matched female wild-type and AQP4 null mice underwent ovariectomy, followed by D-gal administration for 8 wk. AQP4 null mice showed more severe brain oxidative stress, spatial learning and memory deficits, and basal forebrain cholinergic impairment than the wild-type controls. Notably, AQP4 null hippocampus contained more prominent amyloid-β production and loss of synapse-related proteins. These results suggested that ovariectomy and D-gal injection induced oxidative damage results in compensatory increases of AQP4 expression, and deficiency of AQP4 exacerbates brain oxidative stress and memory deficits. Therefore, regulation of astrocyte function by AQP4 may attenuate oxidative damage, offering a promising therapeutic strategy for AD.

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Introduction

Until recently, astrocytes were regarded as passive supportive cells in the central nervous system. This view has changed due to great progress in the field of astrocyte biology. Astrocytes regulate blood flow, energy metabolism and synaptic transmission as well as control fluid, ion and pH homeostasis in the brain (Allen & Barres, 2009). Additionally, they produce neurotrophic factors, growth factors and antioxidants for protecting neurons against a multitude of insults (Barres, 2008). Astrocyte dysfunction contributes to pathogenesis of certain neurological disorders (Seifert et al. 2006). Restoring astrocyte function could lead to a novel therapeutic strategy for epilepsy (David et al. 2009), Alzheimer’s disease (AD) (Fuller et al. 2009) and Parkinson’s disease (PD) (McGeer & McGeer, 2008). Therefore, identifying functional modulators of astrocytes and revealing their novel roles in neurological diseases, are of great significance in theory and practice (Escartin & Bonvento, 2008).

Studies suggest that aquaporin-4 (AQP4) may be a potential molecular target for regulating astrocyte function (Verkman, 2005). AQP4, the most abundant water channel in the brain, is highly expressed in astrocyte processes around brain microvessels (Nielsen et al. 1997). Aside from cerebral water balance (Amiry-Moghaddam & Ottersen, 2003), AQP4 also plays a critical role in other biological functions of astrocytes such as astroglial migration (Auguste et al. 2007),
K⁺ buffering (Binder et al. 2006), glutamate uptake (Zeng et al. 2007), neural signal transduction (Ding et al. 2007), and adult neurogenesis (Kong et al. 2009). Moreover, microarray and functional analyses on cultured human Down syndrome neural progenitor cell lines revealed a new role of AQP4, one that transports reactive oxygen species (ROS) and attenuates oxidative damage induced by exposure to ROS (Esposito et al. 2008). Abnormal expression of AQP4 has been observed in human brain with infarction (Aoki et al. 2003), infection (Aoki-Yoshino et al. 2005), neuromyelitis optica (Misu et al. 2007), epilepsy (Eid et al. 2005), and autism (Fatemi et al. 2008). Therefore, therapeutic strategies targeted at astrocyte function modulated by AQP4 may offer a new therapeutic option for neuropsychological disorders (Fan et al. 2008).

Rodents chronically injected with D-galactose (D-gal) are a well-established animal model for studying the mechanisms of brain ageing and age-related neurodegenerative disorders. Rats, or mice, receiving 50–200 mg/kg D-gal injection per day for 6–12 wk, show memory deficits, brain oxidative stress and energy metabolism disturbance (Gong et al. 2008; Wei et al. 2005; Zhang et al. 2008). Moreover, several hallmark changes associated with AD, including cholinergic degeneration (Lei et al. 2008b), neuronal apoptosis (Cui et al. 2006), amyloid-beta (Aβ) production (Hsieh et al. 2009; Luo et al. 2009), reactive astroglisis (Lei et al. 2008a), and altered expressions of synaptic genes and proteins (Wei et al. 2008; Wu et al. 2008) are also observed in rodents treated with D-gal.

Oestrogen plays a distinctive neuroprotective role in the central nervous system (Maggi et al. 2004). Epidemiological studies indicate that long-term oestrogen deficiency is related to an increased risk of AD (Vegeto et al. 2008). In order to mimic brain oxidative stress and low levels of oestrogen in patients with AD, we established a new rodent model for AD using long-term D-gal injection combined with ovariectomy (OVX) (Hua et al. 2007). Biochemical and pathological alterations of astrocytes are also observed in this model (Hua et al. 2008). Our recent study demonstrated that AQP4 expression is increased in the brain of OVX mice treated with D-gal; however, pathophysiological roles and mechanisms of AQP4 up-regulation remain unclear (Liu et al. 2010).

There is currently a lack of specific modulators or blockers for AQP4, therefore AQP4 gene knockout (AQP4−/−) is an available strategy to address pathophysiological function of AQP4. The present results indicate that AQP4 deficiency exacerbates brain oxidative damage and memory deficits induced by OVX and D-gal injection. These findings reveal a new role of AQP4-mediating antioxidative capability of astrocytes in the brain.

Materials and methods

Animals

AQP4−/− mice in a CD1 genetic background have been established in our laboratory (Fan et al. 2005) and successfully used for investigating pathophysiological roles of AQP4 in several neuropsychological disorders, such as PD (Fan et al. 2008), intracerebral haemorrhage (Tang et al. 2010), depression (Kong et al. 2009), and addiction (Xie et al. 2009). The present study was performed on 5-month-old female AQP4−/− and wild-type (AQP4+/+) mice. Experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University and complied with local and national regulations in accordance with the international standards on animal welfare.

Treatment

The AD model was as described previously (Hua et al. 2007). Briefly, mice were anaesthesitized with 3.5% chloral hydrate (360 mg/kg i.p., Sigma, USA) and underwent bilateral OVX. One week later, D-gal (60 mg/kg d i.p., Sigma), mixed with 0.9% saline was administered intraperitoneally each day for 8 wk. Control mice received the sham operation and were treated solely with 0.9% saline. All groups of mice survived and had similar body weight gain (data not shown).

Morris water maze test

The apparatus of the Morris water maze was a black plastic pool with a diameter of 100 cm and a height of 50 cm. It was housed in a light-controlled room and maintained at a temperature of 22 ± 2 °C. A digital video camera was positioned directly above the pool and connected with a computer-controlled system (Beijing Sunny Instruments Co. Ltd, China) to enable full collection of the swimming pattern, distance and speed.

The training consisted of six consecutive days of testing, with four trials per day. The mouse was placed in the water with its head facing the wall at one of the starting points in a random manner. Timing of the latency to find the platform was started and ended by the experimenter. If the mouse could not reach the platform within 60 s, the experimenter gently assisted it onto the platform and allowed it to remain there for 15 s. The mouse was then dried and returned to the
cage to rest for 15 min before the next trial. During the first 2 d of testing, mice were trained with a visible platform. A cylindrical dark-coloured platform with a diameter of 10 cm was placed 0.5 cm above the surface of water and kept constantly in one of the quadrants. From day 3 hidden platform testing was performed. The platform was moved to the opposite quadrant and submerged 1 cm below the surface of the water. The escape latency, the swim distance and speed, and the swim patterns were analysed. On the last day, 1 h following completion of the training trials, the platform was removed and the mouse was released from the opposite quadrant and allowed to swim for 60 s to determine its search patterns. The percentage of total time spent in each quadrant was also measured. All tests were performed by two independent experimenters who blind to the treatment schedule.

**Preparation of brain tissue**

For biochemical measurement and Western blotting analysis, mice were sacrificed by cervical dislocation and brains quickly removed. The hippocampus was dissected and stored at −80°C for later use. For immunohistochemistry, mice were perfused intracardially with 4% paraformaldehyde. The basal forebrain and the hippocampus were dissected and post-fixed overnight at 4°C. Some hippocampal tissues were processed for paraffin sectioning (5 µm thick). The remaining hippocampus and forebrain tissues were frozen in 40-µm sections. Region definitions of the basal forebrain and hippocampus were based on a mouse brain atlas (Franklin & Paxinos, 1997).

**Biochemical measurement**

The hippocampal tissues were homogenized in cold saline. The homogenate (10%) was centrifuged at 4000 g at 4°C for 10 min. The supernatant was used for measurements of malondialdehyde and reduced glutathione (GSH) contents, plus total superoxide dismutase activities, total antioxidative capabilities and GSH peroxidase activities. All examinations were performed according to the manufacturer’s instructions (Jiancheng Institute of Biotechnology, China). The detailed methods have been described in our previously published report (Lei et al. 2008a).

**Immunohistochemistry**

Forty-µm free-floating or 5-µm dewaxed paraffin sections were incubated overnight at 4°C with rabbit polyclonal anti-AQP4 antibody (1:800, Chemicon, USA), mouse monoclonal anti-choline acetyltransferase (ChAT) antibody (1:500, Chemicon), rabbit polyclonal anti-Aβ A4 protein antibody (1:500, Chemicon), or mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:500, Chemicon), respectively. After incubation with biotinylated IgGs and ABC (Vector, USA), the reaction was visualized with DAB. Slides were washed, dehydrated, and coverslipped with DPX mountant.

**Stereological analysis**

The number and volume of ChAT-immunoreactive (IR) neurons in the medium septum-banda diagonalis complex (MS-DB) were counted using the optical fractionator method with the aid of Stereo-Investigator software (Microbrightfield, USA). Following published methods (Kalesnykas et al. 2004), every fourth section throughout the MS-DB was used for analysis. After outlining the boundaries of the MS-DB on the computer graphic display in each section separately, a point grid was placed randomly over the region to determine the systematic random placement of the optical dissectors. Only immunolabelled perikarya that occurred within the dissector frames or crossed either of the permitted sides of the frames were counted. The total number of ChAT-IR neurons within the three-dimensional optical dissectors throughout the region were then obtained with the stereological software described above. Additionally, the volume of each counted cell was estimated according to the Cavalieri principle (West et al. 1991). Briefly, a vertical line with three perpendicular gridlines were superimposed, and intersections of gridlines with the cell soma were identified (Jensen & Gundersen, 1993). The neuron volume was obtained according to the mean length of these lines using Stereo-Investigator software.

**Astroglial cell counting**

The representative sections containing the dorsal hippocampus were examined in sequence at 200 × magnification by a photomicroscope (Leica Microsystems, Germany). Activated astrocytes were characterized by hypertrophy of the soma and processes and an increase in the expression of GFAP. Resting astrocytes presented a small cell body with thin highly branched processes. The total numbers of GFAP-IR astrocytes within the whole hippocampal regions were counted. The percentages of activated astrocytes were also calculated. Three sections per mouse were averaged to provide a single value for each mouse.
Western blotting

The mouse hippocampi were dissected and homogenized in cold lysis buffer [composed of 50 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS]. The supernatants of the homogenates were subjected to SDS–PAGE, in which the proteins were transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight with synaptophysin (1:1000, Chemicon), post-synaptic density protein-95 (1:1000, Santa Cruz Biotechnology, USA), AQP4 (1:1000) or Aβ A4 protein antibody (1:800). The immunocomplexes were visualized using the ECL detection kit (Amersham Pharmacia Biotech, Canada). Membranes were scanned and analysed using an Omega 16ic Chemiluminescence Imaging System (Ultra-Lum, USA). Signal intensity of each band was normalized to that of β-tubulin.

Statistical analysis

All statistical analyses were performed using SPSS software, version 16.0 (SPSS Inc., USA). The Morris water maze was used to measure spatial memory deficits induced by OVX and D-gal injection. The water maze platform training data were analysed by two-way ANOVA with extent of treatment (OVX plus D-gal injection) as the between-subjects variable, and genotype and treatment (OVX plus D-gal injection) as the within-subject variable, and genotype and treatment. Repeated-measures ANOVA revealed that the visible platform test to determine genotype and learning and memory of the mice. We first performed the visible platform test to determine genotype and learning and memory of the mice. We then evaluated the spatial learning ability of the mice in the hidden platform test. The escape latency and swim distance progressively decreased over 4 d of training in all groups [F(2,22, 97.88) = 630.854, p < 0.001; F(2,618, 115.19) = 307.658, p < 0.001; the degrees of freedom were adjusted by Greenhouse–Geisser correction, respectively] and affected by treatment [F(1, 44) = 104.113, p < 0.001; F(1, 44) = 95.494, p < 0.001, respectively], genotype [F(1, 44) = 9.168, p = 0.004; F(1, 44) = 8.240, p = 0.006, respectively] and treatment × genotype interaction [F(1, 44) = 5.644, p = 0.022; F(1, 44) = 6.349, p = 0.015, respectively]. Post-hoc analyses revealed that, on each training day, both AQP4+/− and AQP4−/− mice used long escape latency and swim distance compared to AQP4+/−-treated mice (p < 0.05), whereas there was no difference between AQP4−/− and AQP4−/−–OVX plus D-gal-treated groups needed more time and distance to find the hidden platform than the two vehicle controls (p < 0.01). Furthermore, AQP4−/−-treated mice used long escape latency and swim distance compared to AQP4+/−-treated mice (p < 0.05), whereas there was no difference between AQP4+/− and AQP4−/−–control groups (p > 0.05) (Fig. 1a, b). Swim speed was not affected by treatment [F(1, 44) = 0.151, p = 0.699] or genotype [F(1, 44) = 0.760, p = 0.388] (Fig. 1c), further indicating that the swim ability was intact and not related to the spatial learning defect caused by OVX and D-gal treatment.

We also determined whether delays in finding the hidden platform were associated with abnormal search pattern and deficit in OVX and D-gal-treated mice. Figure 1d shows the representative tracing at the fourth trial for each group from each test day. On the first day of the hidden platform training (day 3 of training trials), both AQP4+/− and AQP4−/−–control mice mainly swam within the inner portion of the pool with weaving or looping search patterns to find the platform. This highly suggested that the control mice had learned that the hidden platform was not located around the perimeter of the maze after training three times. In contrast, OVX and D-gal-treated mice, especially AQP4−/− mice, swam randomly within the entire pool with a high proportion in the outer portion of the pool, indicating that these mice found the platform due to chance. Even up to the last trial on day 6, the outer portion searching strategies were still observed in AQP4+/−- and AQP4−/−-treated mice, but rarely in vehicle controls.

Results

AQP4 deficiency aggravates spatial learning and memory deficits induced by OVX and D-gal injection

The Morris water maze was used to measure spatial learning and memory of the mice. We first performed the visible platform test to determine genotype and treatment effects on motor ability and visual-spatial function. Repeated-measures ANOVA revealed that escape latency and swim distance were affected by training days [F(1, 44) = 140.115, p < 0.001; F(1, 44) = 52.459, p < 0.001, respectively] but not by treatment [F(1, 44) = 0.665, p = 0.19; F(1, 44) = 0.343, p = 0.561, respectively] or genotype [F(1, 44) = 0.003, p = 0.956; F(1, 44) = 1.086, p = 0.303, respectively] (Fig. 1a, b). Moreover, there was no difference in swim speed in all groups during the 2 d of training test [F(1, 44) = 1.459, p = 0.234] (Fig. 1c). These results suggest that neither OVX plus D-gal treatment for 8 wk nor AQP4 deficiency causes apparent motor and/or visual deficits in the adult mice.

Moreover, there was no difference in swim speed in all groups during the 2 d of training test [F(1, 44) = 1.459, p = 0.234] (Fig. 1c). These results suggest that neither OVX plus D-gal treatment for 8 wk nor AQP4 deficiency causes apparent motor and/or visual deficits in the adult mice.

We then evaluated the spatial learning ability of the mice in the hidden platform test. The escape latency and swim distance progressively decreased over 4 d of training in all groups [F(2,22, 97.88) = 630.854, p < 0.001; F(2,618, 115.19) = 307.658, p < 0.001; the degrees of freedom were adjusted by Greenhouse–Geisser correction, respectively] and affected by treatment [F(1, 44) = 104.113, p < 0.001; F(1, 44) = 95.494, p < 0.001, respectively], genotype [F(1, 44) = 9.168, p = 0.004; F(1, 44) = 8.240, p = 0.006, respectively] and treatment × genotype interaction [F(1, 44) = 5.644, p = 0.022; F(1, 44) = 6.349, p = 0.015, respectively]. Post-hoc analyses revealed that, on each training day, both AQP4+/− and AQP4−/−–OVX plus D-gal-treated groups needed more time and distance to find the hidden platform than the two vehicle controls (p < 0.01). Furthermore, AQP4−/−-treated mice used long escape latency and swim distance compared to AQP4+/−-treated mice (p < 0.05), whereas there was no difference between AQP4+/− and AQP4−/−–control groups (p > 0.05) (Fig. 1a, b). Swim speed was not affected by treatment [F(1, 44) = 0.151, p = 0.699] or genotype [F(1, 44) = 0.760, p = 0.388] (Fig. 1c), further indicating that the swim ability was intact and not related to the spatial learning defect caused by OVX and D-gal treatment.

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To assess whether a mouse had learned the location of the hidden platform, the probe test was conducted 1 h following completion of the training trials on day 6. Figure 1e shows the percentage of time spent in each quadrant. Two-way ANOVA revealed significant effects of treatment \(F(1, 44) = 209.255, p < 0.001\), genotype \(F(1, 44) = 8.253, p = 0.005\) and their interaction \(F(1, 44) = 7.679, p = 0.007\) on time spent in the target quadrant. Both AQP4\(^{+/+}\) and AQP4\(^{-/-}\) OVX plus D-gal-treated groups spent lower percentage times in the target quadrant compared to AQP4\(^{+/+}\) and AQP4\(^{-/-}\) Sham+Sal groups. The black circles indicate the position of the escape platform. Arrowheads indicate the position for the beginning of the swim. Data represent means \(\pm\) S.E.M. from 12 mice per group. * \(p < 0.05\), ** \(p < 0.01\), within genotype comparisons; # \(p < 0.05\), ## \(p < 0.01\), between genotype comparisons.

Fig. 1. Morris water maze test. (a) The mean escape latency, (b) swim distance to the platform, and (c) swim speed in the visible platform test (days 1–2) and hidden platform test (days 3–6). (e) Percentage of time spent in each quadrant in the probe test. (d) and (f) Tracings of the typical swim patterns in the hidden platform test and in the probe test, respectively. Both AQP4\(^{+/+}\) and AQP4\(^{-/-}\) control mice tended to have smaller and more focused loops to reach the hidden platform from training days 3–6 and had a strong preference for the target quadrant in the probe test. In contrast, both AQP4\(^{+/+}\) and AQP4\(^{-/-}\) OVX plus D-gal-treated mice tended to search in a bigger area of the pool without an apparent preference for the target quadrant during the training and probe trials. The black circles indicates the position of the escape platform. Arrowheads indicates the position for the beginning of the swim. Data represent means \(\pm\) S.E.M. from 12 mice per group. * \(p < 0.05\), ** \(p < 0.01\), within genotype comparisons; # \(p < 0.05\), ## \(p < 0.01\), between genotype comparisons.
results from at least three independent experiments. Data represent means ± S.E.M. from four mice per group. ** p < 0.01, within genotype comparisons.

in the target quadrant while higher percentage time in the two adjoining quadrants than the two vehicle control groups (p < 0.05; Fig. 1b). Moreover, AQP4+/− -treated mice spent less time in the target quadrant than AQP4+/+ -treated mice (p < 0.05).

Representative tracings provided further insight into memory deficit in OVX and D-gal-treated mice. As shown in Fig. 1f, control mice swam directly to, and searched intently in, the target quadrant after they were placed in the water from the opposite quadrant. By contrast, OVX plus D-gal-treated mice used non-spatial systematic or even repetitive looping paths to reach the target quadrant and then moved towards other quadrants without extensive searching. Together, these behavioural data demonstrate that there are spatial learning and memory defects in OVX plus D-gal-treated mice, and that these impairments are greater in AQP4+/− mice.

Increased AQP4 expression in the hippocampus of OVX and D-gal-treated wild-type mice

We investigated AQP4 expression in the basal forebrain (data not shown) and hippocampus in OVX plus D-gal-treated mice. The intensity of AQP4 immunostaining in the hippocampus was higher in AQP4+/−-treated mice than AQP4+/+ controls (Fig. 2a). No immunoreactivity was observed in the hippocampus of both AQP4+/− mice (Fig. 2a). Consistently, AQP4 protein expression level in the hippocampus of AQP4+/+ -treated mice was nearly three times higher than that in AQP4+/− controls (p < 0.01; Fig. 2b).

AQP4 deficiency increases brain oxidative stress induced by OVX and D-gal injection

It has been well demonstrated that long-term exposure of mice or rats to D-gal results in brain oxidative stress (Gong et al. 2008; Zhang et al. 2008). We determined that whether AQP4 deficiency increased brain oxidative stress by measuring levels of malondialdehyde (MDA), a marker of lipid peroxidation, and levels of antioxidative indexes including total superoxide dismutase activities (T-SOD) and total antioxidative capabilities (T-AOC) in the hippocampus of the four groups. Two-way ANOVA revealed significant effects of treatment [F_{T−AOC}(1, 20) = 69.231, p < 0.001; F_{T−SOD}(1, 20) = 82.387, p < 0.001; F_{MDA}(1, 20) = 71.934, p < 0.001], genotype [F_{T−AOC}(1, 20) = 4.651, p = 0.043; F_{T−SOD}(1, 20) = 5.249, p = 0.033; F_{MDA}(1, 20) = 10.587, p = 0.004], and their interaction [F_{T−AOC}(1, 20) = 9.266, p = 0.006; F_{T−SOD}(1, 20) = 6.205, p = 0.022; F_{MDA}(1, 20) = 6.059, p = 0.023] on T-AOC, T-SOD and MDA levels. There was no significant difference in baseline levels of the above parameters between AQP4+/− and AQP4+/+ control mice (p > 0.05). Both AQP4+/− and AQP4+/+ OVX plus D-gal-treated mice showed decreases in T-AOC and T-SOD activities, but an increase in MDA levels (p < 0.05; Fig. 3).
Our previous study showed loss of basal forebrain cholinergic neurons in OVX plus D-gal-treated rats. Both AQP4+/+ and AQP4–/– mice treated with OVX and D-gal showed a significant decrease in the number of ChAT-IR neurons (p < 0.01), whereas loss was more evident in AQP4–/–-treated mice compared to AQP4+/+ treated mice (p < 0.01; Fig. 4a, b).

Moreover, increased MDA levels and decreased T-AOC and T-SOD activities were more significant in AQP4–/–-treated mice than AQP4+/+ -treated mice (p < 0.05).

**AQP4 deficiency exacerbates degeneration of basal forebrain cholinergic neurons induced by OVX and D-gal injection**

Our previous study showed loss of basal forebrain cholinergic neurons in OVX plus D-gal-treated rats (Hua et al. 2007). We further addressed whether the exacerbated spatial learning and memory deficits with loss of synaptic proteins, the expression levels of presynaptic vesicle protein synaptophysin (SYP) and post-synaptic density protein-95 (PSD-95) in the hippocampus were determined by Western blotting. Treatment [F_{Sy}p(1, 12) = 167.673, p < 0.001; F_{PSD-95}(1, 12) = 78.062, p < 0.001], genotype [F_{Sy}p(1, 12) = 17.276, p = 0.001; F_{PSD-95}(1, 12) = 6.905, p = 0.022], and their interaction [F_{Sy}p(1, 12) = 5.497, p = 0.037; F_{PSD-95}(1, 12) = 7.942, p = 0.016] affected the expressions of SYP and PSD-95 (Fig. 5). There was no difference in baseline expressions of SYP and PSD-95 between AQP4+/+ and AQP4–/– control mice (p > 0.05). Decreases in these two synaptic proteins were observed in both AQP4+/+ and AQP4–/–-treated mice (p < 0.01), but were more evident in AQP4–/–-treated mice (p < 0.01).

**AQP4 deficiency increases accumulation of Aβ in the hippocampus of OVX plus D-gal-treated mice**

It is known that soluble Aβ impairs synaptic plasticity (Shankar et al. 2008) and disrupts synapse composition, shape, and density in AD brain (Lacor et al. 2007). To confirm the interrelations among brain oxidative stress, Aβ formation and synapse disruption, we compared levels of Aβ expression in the hippocampus among the four groups. There was only a low baseline level of Aβ immunoreactivity in AQP4+/+ and AQP4–/– control mice. Increased
Aβ immunostaining was observed in neurons in the hippocampal dentate gyrus of both AQP4+/+ and AQP4−/−-treated mice, but much stronger staining of Aβ was seen in AQP4−/−-treated mice (Fig. 6a). Consistently, two-way ANOVA analysis of Western blotting results revealed significant effects of treatment \[F(1, 12) = 54.031, p < 0.001\], genotype \[F(1, 12) = 6.835, p = 0.023\], and their interaction \[F(1, 12) = 6.960, p = 0.022\] on expression levels of Aβ. Aβ levels were much higher in the hippocampus of AQP4−/−-treated mice than in AQP4+/+ -treated mice \((p < 0.01; \text{Fig. 6b})\).

**AQP4 deficiency does not alter astrogliosis in the hippocampus of OVX plus D-gal treated mice**

Reactive astrogliosis is a pathological hallmark of neurodegenerative disorders including AD (Fuller et al. 2009). We performed GFAP immunohistochemistry in order to investigate activation of astrocytes in the hippocampus of the four groups. Activated astrocytes with hypertrophic cell bodies and intensely stained processes were dramatically increased in both AQP4−/− and AQP4+/+ -treated mice (Fig. 7a). Two-way ANOVA revealed a significant effect of treatment on the total number of GFAP-IR astrocytes \[F(1, 20) = 67.784, p < 0.001\] and the percentage of activated astrocytes \[F(1, 20) = 256.944, p < 0.001\], but not genotype \[F_{\text{number}}(1, 20) = 2.494, p = 0.130; F_{\text{percentage}}(1, 20) = 0.315, p = 0.581\] or treatment × genotype interaction \[F_{\text{number}}(1, 20) = 1.021, p = 0.324; F_{\text{percentage}}(1, 20) = 0.105, p = 0.755\]. Treated mice had more GFAP-IR astrocytes and a larger percentage of activated astrocytes than untreated mice,
but there was no significant difference between AQP4+/– and AQP4+/- mice (Fig. 7b, c).

Moreover, biochemical analysis demonstrated that AQP4 deficiency failed to change the decline in reduced GSH contents and GSH peroxidase activities in OVX plus D-gal-treated mice (Supplementary Fig. 1, available online). These results suggest that impaired antioxidant capacity of astrocytes is involved in this AD model (Hua et al. 2008), but AQP4 deficiency has no detrimental effects on GSH production from astrocytes.

Discussion

Numerous studies have highlighted the critical role of AQP4 in the pathogenesis of brain oedema (Papadopoulos & Verkman, 2007) and neuromyelitis optica (Jarius et al. 2008). Nevertheless, there is limited literature investigating expressions of AQP’s in AD patients as well as AD animal models (Pérez et al. 2007; Wilcock et al. 2009). Furthermore, pathophysiological effects of AQP4 on progress of AD have yet to be determined. In the present study, we demonstrated that increased expression of AQP4 and AQP4 deficiency aggravated oxidative damage and memory deficits in OVX plus D-gal-treated mice which serves as a mouse model of sporadic AD.

D-gal, a reducing sugar in the body, can be metabolized by D-galactokinase and galactose-1-phosphate uridylyltransferase at normal concentration. However, at high levels, beyond the capacity of those two enzymes, it is converted into aldose and hydroperoxide by galactose oxidase, resulting in the generation of a superoxide anion and oxygen-derived free radicals (Gong et al. 2008). In addition, accumulated D-gal can react with the free amino groups of proteins and peptides to form advanced glycation end products, leading to osmotic stress and ROS production (Song et al. 1999). Thus, long-term injection of D-gal can mimic brain oxidative stress-induced memory deficits. In the present study, abnormalities of brain oxidative/antioxidative parameters and behaviour performances in OVX plus D-gal-treated mice confirm this notion. Moreover, we demonstrated that AQP4 deficiency exacerbated these biochemical and behavioural impairments in OVX plus D-gal-treated mice.

Memory deficits in OVX plus D-gal-treated mice may be attributed to synaptic protein loss in the hippocampus and cholinergic degeneration in the MS-DB. Both SYP and PSD-95 are useful markers to investigate the correlation between synaptic protein loss and cognitive decline in AD (Sze et al. 1997). Decreases in the expression of synaptic proteins including SYP and PSD-95 were observed in the hippocampus following long-term injection of D-gal and reversed by purple sweet potato colour that has antioxidant capabilities (Wu et al. 2008). In agreement with these results, OVX mice injected with D-gal had reduced levels of SYP and PSD-95 in the hippocampus. Furthermore, AQP4 absence causes more prominent loss of these synaptic proteins, consistent with more severe memory deficits in AQP4+/- OVX plus D-gal-treated mice.

The MS-DB is a major source of cholinergic projections to the hippocampus, playing a crucial role in spatial learning and memory in the mammalian brain (Everitt & Robbins, 1997). Degeneration of the septo-hippocampal cholinergic system has been linked to the cognitive impairment in brain ageing and AD (Schliebs & Arendt, 2006). Additionally, this circuit is susceptible to oxidative damage (McKinney & Jacksonville, 2005). Published results from three independent laboratories, including our own, have reported that chronic exposure of D-gal impairs the basal forebrain cholinergic system in rodents (Lei et al. 2008b; Lu et al. 2010; Zhong et al. 2009). Notably, the survival of the cholinergic forebrain neurons seems to be associated with the levels of endogenous oestrogen...
Gibbs, 1998). Our previous study suggested that long-term ovarian hormone deprivation and D-gal injection have synergistic effects on the acceleration of cholinergic and memory deficits (Hua et al., 2007). We further reported that pathological changes and decreased antioxidant capability of astrocytes are involved in degeneration of cholinergic terminals in the hippocampus of OVX rats injected with D-gal (Hua et al., 2008). In the present study, we provide evidence that AQP4 deficiency aggravated impairment of the septohippocampal cholinergic circuit in this mouse model. Together, these results indicate that restoring neuroprotective potential of astrocytes by targeted AQP4 might be a promising therapeutic strategy to reverse cholinergic and memory deficits in AD patients.

Western blot and immunohistochemical analyses revealed a prominent increase in AQP4 expression in wild-type treated mice. We considered that AQP4 up-regulation may be mainly attributed to advanced glycation end products and ROS induced by D-gal and OVX. Earlier studies showed that hyperosmotic stress induced by mannitol increases expression of AQP4 and AQP9 in cultured rat astrocytes, and intraperitoneal infusion of mannitol up-regulates AQP4 and AQP9 expression in the rat brain cortex (Arima et al. 2003). Moreover, microarray analyses of human Down syndrome neural progenitor cell lines demonstrated that overexpression of genes such as S100b and amyloid precursor protein increases production of ROS and activation of stress response protein kinases, leading to compensatory increases in AQP4 expression. Also, up-regulation of AQP4 expression can be induced by direct exposure to ROS (Esposito et al. 2008). Based on more serious cholinergic degeneration, synaptic protein loss and Aβ accumulation in AQP4−/− OVX plus D-gal-treated mice, we further propose that increased AQP4 expression may serve to promote ROS clearance from brain parenchyma and attenuate neuronal oxidative damage induced by OVX and D-gal injection. This conclusion is also supported by the evidence from functional analyses of human Down syndrome neural progenitor cell lines that siRNA inhibition of AQP4 results in elevated levels of ROS following S100β exposure, causing increased programmed cell death (Esposito et al. 2008). In addition, a large body of evidence implicates a critical role of AQPs in transport and metabolic degradation of hydrogen peroxide in plant cells (Bienert et al. 2007; Dynowski et al. 2008). Collectively, these results provide new evidence in vivo that AQP4 is up-regulated under brain oxidative conditions, and may serve to transport ROS from the central nervous system to the peripheral circulation.
consequently protecting neurons against oxidative damage.

It is interesting to note that AQP4+/− OVX plus D-gal-treated mice showed more severe Aβ accumulation, and synapse-related protein loss but not more prominent astrogliosis in the hippocampus than wild-type controls. In agreement with the present results, the previous study from our laboratory indicated that AQP4 deficiency aggravates loss of dopaminergic neurons but attenuates astroglial proliferation in the substantia nigra pars compacta after administration of MPTP (Fan et al. 2008). In addition, AQP4 null mice also show impaired migration of reactive astrocytes towards the lesion site and glial scar formation following cortical stab injury (Auguste et al. 2007). These results reveal a role of AQP4 for reactive astrogliosis; however, the underlying mechanisms warrant further investigation.

In conclusion, the present study demonstrates that AQP4 deficiency aggravates memory deficits and brain oxidative damage, including the basal forebrain cholinergic degeneration, loss of synaptic proteins, and Aβ accumulation in the hippocampus of AD mice established by long-term D-gal injection and ovarian hormone deprivation. These results reveal a previously unexpected role for AQP4 in mediating brain oxidative stress. Therefore, regulation of astrocyte function with AQP4 may attenuate oxidative damage, offering a promising therapeutic strategy for AD.
Note

Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

References


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