Aquaporin-4 Expression in Post-Traumatic Syringomyelia

Sarah J. Hemley,¹ Lynne E. Bilston,^{2,3} Shaokoon Cheng,^{2,4} Jing Ning Chan,⁵ and Marcus A. Stoodley¹

Abstract

Aquaporin-4 (AQP4) is an astroglial water channel protein that plays an important role in the transmembrane movement of water within the central nervous system. AQP4 has been implicated in numerous pathological conditions involving abnormal fluid accumulation, including spinal cord edema following traumatic injury. AQP4 has not been studied in post-traumatic syringomyelia, a condition that cannot be completely explained by current theories of cerebrospinal fluid dynamics. Alterations of AQP4 expression or function may contribute to the fluid imbalance leading to syrinx formation or enlargement. The aim of this study was to examine AQP4 expression levels and distribution in an animal model of post-traumatic syringomyelia. Immunofluorescence and western blotting were used to assess AQP4 and glial fibrillary acidic protein (GFAP) expression in an excitotoxic amino acid/arachnoiditis model of post-traumatic syringomyelia in Sprague-Dawley rats. At all time-points, GFAP-positive astrocytes were observed in tissue surrounding syrinx cavities, although western blot analysis demonstrated an overall decrease in GFAP expression, except at the latest stage investigated. AQP4 expression was significantly higher at the level of syrinx at three and six weeks following the initial syrinx induction surgery. Significant increases in AQP4 expression also were observed in the upper cervical cord, rostral to the syrinx except in the acute stage of the condition at the three-day time-point. Immunostaining showed that AQP4 was expressed around all syrinx cavities, most notably adjacent to a mature syrinx (six- and 12-week time-point). This suggests a relationship between AQP4 and fluid accumulation in post-traumatic syringomyelia. However, whether this is a causal relationship or occurs in response to an increase in fluid needs to be established.

Key words: aquaporin-4; post-traumatic syringomyelia; spinal cord injury

Introduction

TRAUMATIC SPINAL CORD INJURY is a devastating condition affecting approximately 13,000 people each year in the United States.^{1–3} Of these, up to one third will develop post-traumatic syringomyelia, a serious disease characterized by the formation of fluid-filled cysts or cavities (syrinxes) within the spinal cord. As a syrinx enlarges, it causes damage to the surrounding spinal tissue, leading to pain and/or an additional decline in motor and sensory function. The underlying pathogenesis of syringomyelia is not completely understood, and outcomes from surgical treatments are often unsatisfactory, with clinical studies reporting that only ~50% of patients show improvement.^{4–8}

Current theories of syrinx pathogenesis are based predominantly on altered cerebrospinal fluid (CSF) dynamics.^{9,10} In the case of post-traumatic syringomyelia, it is thought that an obstruction in the subarachnoid space occurs at the time of the traumatic injury or later when arachnoid scarring develops. It is assumed that CSF flows into syrinx cavities from the subarachnoid space surrounding

the cord, with increased flow occurring at the point of obstruction.^{11,12} Since the pressure inside the syrinx and spinal cord have been shown to sometimes exceed subarachnoid space pressure^{13,14} and fluid cannot flow against a pressure gradient, it is thought that CSF cannot be the only source of fluid. There may be additional sources of fluid or other disturbances to fluid homeostasis. There is increasing support for the theory that syrinx enlargement is due, at least in part, to extracellular fluid accumulation.^{15,16} This could be due to tissue destruction and damage to capillaries and venules, allowing plasma filtrate to pass across the blood-spinal cord barrier and into the cord. There is evidence that the blood-spinal cord barrier is impaired in an animal model of post-traumatic syringomyelia even three months after the initial cyst formation.¹⁷

The water channel protein aquaporin-4 (AQP4) facilitates transmembrane water movement in the central nervous system. AQP4 has been implicated in a wide range of pathological conditions involving abnormal water accumulation within the brain and spinal cord.^{18–21} Experimental evidence suggests that an increase in AQP4 expression may contribute to increased water content

¹The Australian School of Advanced Medicine, Macquarie University, Sydney, New South Wales, Australia.

²Neuroscience Research Australia, Sydney, New South Wales, Australia.

³Prince of Wales Clinical School, ⁴School of Medical Science, University of New South Wales, Sydney, New South Wales, Australia.

⁵Gosford Hospital, New South Wales, Sydney, New South Wales, Australia.

TABLE 1. EXPERIMENTAL GROUPS FOR IMMUNOHISTOCHEMISTRY: SURGICAL PROCEDURE AND SURVIVAL TIME IN EXPERIMENTAL RATS

Experimental group	Initial operation	No. of animals at each survival point				
		3 days	1 wk	3 wks	6 wks	12 wks
Control	Laminectomy only	1	1	1	1	1
Sham-injected control	4 intraparenchymal injections of saline	1	1	1	1	1
Syrinx induction	4 intraparenchymal injections of quisqualic acid & subarachnoid kaolin	3	3	3	3	3

following spinal trauma. A spinal cord compression injury model in AQP4 knockout mice found that AQP4 deficiency resulted in improved neurological outcome, decreased neuronal death, less myelin vacuolation, reduced spinal cord swelling, and reduced intraparenchymal pressure.²² A study of AQP4 expression in a rat model of spinal cord injury demonstrated a decrease in AQP4 in the acute stages of spinal cord injury followed by a marked increase in the chronically injured cord. Water content remained significantly higher in the early and late stages of disease in injured cords, compared with controls.²³

The changes in AQP4 expression following spinal cord injury and the possible causal relationship between AQP4 and spinal cord swelling demonstrated by Nesic and colleagues and Saadoun and associates^{22,23} point to the need for further study into AQP4 and its effect on disorders associated with spinal injury such as post-traumatic syringomyelia. We hypothesized that AQP4 may be involved in the initial stages of syrinx formation or in subsequent syrinx enlargement.

Methods

Following ethical approval from the Animal Care and Ethics Committee of the University of New South Wales and Macquarie University, 48 male Sprague-Dawley rats aged 6–10 weeks old and weighing 349 ± 65 g (mean $\pm$ standard deviation) were divided into five experimental groups for immunohistochemistry (Table 1) and western blotting experiments (Table 2). Experimental groups consisted of three animals undergoing a syrinx induction procedure (described below). Controls were either three normal animals (western blotting), or one laminectomy-only control (immunohistochemistry) and one sham-injected control animal that received four spinal cord intraparenchymal injections of $0.5 \mu\text{L}$ saline containing 1% Evans blue (Sigma-Aldrich, St. Louis, Missouri). AQP4 expression was investigated after three days, or at one week, three weeks, six weeks, or 12 weeks following the initial operation. All procedures were performed in a sterile field under general anesthesia induced with 4% isoflurane in oxygen and maintained with 2% isoflurane through a nose cone, which was increased as required to maintain an adequate level of anesthesia.

Syrinx induction

The excitotoxic and arachnoiditis model of post-traumatic syringomyelia has been described previously.^{11,24} Animals were placed prone, and the skin shaved and prepared with povidone iodine. A midline incision was made over the cervicothoracic junction and a laminectomy was performed from C7 to T1. A 29-gauge needle was used to puncture the meninges. A glass-tipped, $5 \mu\text{L}$ syringe (SGE International Pty Ltd., Austin, Texas) held in a stereotactic micromanipulator was then used to infiltrate four $0.5 \mu\text{L}$ injections of 24 mg/mL quisqualic acid (Tocris Cookson, Bristol, UK) and 1% Evans blue. Injections were delivered into the dorsal cord parenchyma along the right dorsal nerve rootlets between the rostral C8 and caudal T1 levels. Five microliters of 250 mg/mL kaolin (Sigma-Aldrich) were then injected into the subarachnoid space to produce arachnoiditis. Wounds were closed with a single layer silk suture. Analgesia was administered post-operatively and the animals were allowed food and water ad libitum. Any excessive weight loss (20 % or more), limb weakness, or signs of over-self grooming were recorded.

Tissue collection, processing and immunohistochemistry

The animals were rapidly perfused by intracardiac injection of 5,000 IU heparin in 1 mL of saline, followed by 500 mL of 4% paraformaldehyde (Lancaster Synthesis, Pelham, New Hampshire) in 0.1 M phosphate buffer (pH 7.4) under a constant pressure of 120 mm Hg. The spinal cord was dissected out and post-fixed in 2% paraformaldehyde in 0.1 M phosphate buffer. Spinal cord segments C7 to T2 were paraffin-embedded, and transverse tissue slices $5\text{--}10 \mu\text{m}$ thickness were cut and mounted on slides and left to dry overnight at 37°C . Spinal cord sections were deparaffinized, rehydrated, and antigen retrieval was performed using 0.01 M citrate buffer (pH 6.0). Sections were blocked in 15% normal horse serum (NHS) in phosphate-buffered saline (PBS) pH 7.45 for 60 min. The sections were then incubated with anti-AQP4 antibody (1:250; AB3594 Chemicon, Temecula, California) and anti-glial fibrillary acidic protein (GFAP) antibody (1:800; MAB360) overnight at 4°C , followed by secondary anti-rabbit IgG Alexa Fluor 488 and anti-mouse IgG Alexa Fluor 594 (Molecular Probes, Eugene,

TABLE 2. EXPERIMENTAL GROUPS FOR WESTERN BLOTTING. SURGICAL PROCEDURE AND SURVIVAL TIME IN EXPERIMENTAL RATS

Experimental group	Initial operation	No. of animals at each survival point				
		3 days	1 wk	3 wks	6 wks	12 wks
Normal Control	None	3*	0	0	0	0
Syrinx induction	4 intraparenchymal injections of quisqualic acid & subarachnoid kaolin	3	3	3	3	3

*Three normal control (non-operated) animals were used. Since there is no surgery involved, the same three animals were used for each time-point.

Oregon) diluted in 4% NHS/PBS 1:400 for 2 h. The sections were coverslipped with fluorescent mounting medium (DAKO, Carpinteria, California). Omission of the primary antibody was used for negative controls. Fluorescent images were obtained using a digital camera (Zeiss Z1, Gottingen, Germany), and processed using Zeiss Axiovision software.

Protein extraction

The animals were rapidly perfused by intracardiac injection with 180 mL of ice cold 0.1 M phosphate buffer (pH 7.4) containing 5000 IU heparin. Spinal cord segments were dissected on ice and immediately frozen on dry ice. Samples were stored at -80°C until homogenization. The tissue samples were then placed in lysing matrix tubes containing ceramic spheres and homogenized in 200 μL of ice cold lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and containing protease and phosphatase inhibitors [Calbiochem, San Diego, California]) in a FastPrep[®]-24 tissue and cell homogenizer (MP Biomedicals, Solon, Ohio) for 20 sec at 4.0 m/s. After vortexing for 5 sec and centrifugation at 8000 rpm for 8 min at 4°C , the whole cell protein supernatant fraction was collected. Protein concentrations were determined using a Bicinchoninic acid assay (bovine serum albumin; Pierce, Rockford, Illinois).

Electrophoresis and western blotting

Samples containing 25 μg of protein and 4X NuPAGE[®] LDS sample buffer and 10X NuPAGE[®] reducing agent (Invitrogen, Carlsbad, California) were heated at 70°C for 10 min, and separated on a 10% NuPAGE Novex Bis-Tris Gel. Samples were immunoblotted on to a polyvinylidene difluoride membrane using the iBlot[®] dry blotting system (Invitrogen, 0.2 μm). Membranes were blocked with 5% (w/vol) non-fat dry milk in tris-buffered saline (TBS) with Tween-20 for 60 min at room temperature and incubated with the primary antibody at 4°C overnight, washed three times in TBS containing 0.1% (w/vol) Tween-20 and then incubated with HRP-conjugated anti-mouse (1:10,000) or anti-rabbit (1:10,000) secondary antibody (Sigma-Aldrich) and visualized by enhanced chemiluminescence (ECL; RPN2232; Amersham Biosciences, UK). The following primary antibodies were used: rabbit anti-AQP4 (Abcam AB46182, 1:4000); mouse anti-GFAP (Chemicon MAB360, 1:60,000); and rabbit anti-Actin (Sigma-Aldrich A5060, 1:3000) as a loading control. To ensure specific binding of the anti-AQP4 antibody, an immunizing peptide blocking experiment was performed using an AQP4 peptide (Abcam AB46181). Immunoblotting produced three AQP4 immunopositive bands, two robust bands at ~ 37 kDa and ~ 45 kDa, and a faint band at ~ 28 kDa (Fig. 1).

Signal intensity was quantified using Image-J software (developed at the U.S. National Institutes of Health, available at rsb.info.nih.gov/nihi-image). Specifically, a fixed area was used to separately measure signal intensity from i) the region encompassing the ~ 37 kDa AQP4 band; ii) the GFAP band at ~ 51 kDa; and iii) the Actin band at ~ 42 kDa. The AQP4 and GFAP signal intensities were normalized to Actin. Where possible, relative differences between samples were assessed on the same blots or using simultaneously processed gels with identical film exposure times. Spinal cord segments investigated for each animal were: C2 to C3, C7 to C8, T1 to T2 and T6 to T7.

Statistics

AQP4 expression in control and syrinx animals was compared using univariate analysis of variance with post-hoc Bonferroni to adjust for multiple comparisons. A probability value <0.05 was considered statistically significant. Data are presented as the mean $\pm$ SEM. Software used included Excel (Microsoft, Redmond, Washington) and GraphPad Prism 5 (GraphPad Software, La Jolla, California).

FIG. 1. Representative western blot for aquaporin-4 (AQP4) showing 2 bands, one at ~ 37 kDa and another at ~ 45 kDa, in C7 spinal cord sections from control (C) and syrinx (S) animals, 3 weeks post-surgery. The negative control obtained after blocking with the AQP4 peptide is also shown (-ve).

Results

The intraspinal injection of quisqualic acid and subarachnoid kaolin injection produced a noncommunicating extracanalicular syrinx cavity in all 15 animals. Transient right forelimb weakness was commonly observed following the syrinx induction surgery. Animals recovered within three days. Over-grooming of the right fore-limb was observed in three animals.

AQP4 and GFAP expression pattern in controls

In control (laminectomy-only) animals, AQP4 was primarily expressed in gray and white matter astrocytes (Fig. 2). In the gray matter, AQP4 was strongly expressed surrounding capillaries, and more diffusely throughout the gray matter (Fig. 2B). Co-localization of GFAP and AQP4 was very limited in the gray matter; however, GFAP expression was observed in glial processes adjacent to astrocytic endfeet labeled with AQP4. AQP4 was also expressed, although not as strongly, in ependymal cells lining the central canal (Fig. 2C). In the white matter AQP4 was expressed in radial astrocytes, predominantly at the glia limitans externa and co-localized with GFAP positive astrocytes (Fig. 2D). Capillaries and ependymal cells were identified by their characteristic morphology.

In sham-injected controls a small cavity was observed at three days, and at one week, six weeks, and 12 weeks following the intraparenchymal injection of saline and Evans blue. These cavities were restricted to the dorsal horn gray matter or dorsal white matter (Fig. 3A). At three weeks after induction a larger cavity was

FIG. 2. (A) Immunolocalization of aquaporin-4 (AQP4; green) and glial fibrillary acidic protein (GFAP; red) in control (laminectomy-only) rat spinal cord. Co-localization of AQP4 and GFAP shown in yellow. Boxes in A indicate areas shown at higher magnifications in boxes B-D. (B) AQP4 is expressed around capillaries (arrows) in gray matter; (C) in ependymal cells (arrow) and gray matter (arrow head) surrounding the central canal; and (D) at the glia limitans externa (arrow heads). CC = central canal. Scale bar is (A) 500 μ m; (B-D) 50 μ m. Color image is available online at www.liebertpub.com/neu

observed in the dorsal white matter, extending into dorsal gray matter. This demonstrates that the spinal injection itself causes some mechanical damage and edema in the spinal cord. GFAP positive astrocytes surrounded the cavity and AQP4 was expressed around the cavity at all time-points (Fig. 3 B-E).

AQP4 and GFAP expression pattern in post-traumatic syringomyelia

At all time-points (three days, one week, three weeks, six weeks, and 12 weeks) investigated, GFAP-positive astrocytes were observed around the syrinx cavity. AQP4 expression was observed directly adjacent to the cavity at all time-points, following the same pattern as GFAP expression (Fig. 4). At the earlier time-points—three days, one week, and three weeks following syrinx induction—AQP4 expression levels surrounding the syrinx appeared similar or lower than expression away from the syrinx. From six weeks after induction, however, AQP4 expression surrounding the syrinx cavity had increased, compared with the surrounding tissue.

At 12 weeks after surgery, AQP4 expression was higher surrounding the syrinx, appearing to be similar to or greater than GFAP expression surrounding the syrinx (Fig. 5).

Western blot analysis of AQP4 and GFAP in post-traumatic syringomyelia

There was an increase in AQP4 expression in syrinx animals, compared with control animals at all time-points except three days following the syrinx induction procedure (Fig. 6). However, this was not observed at all spinal levels. At one week and 12 weeks after syrinx induction, AQP4 had significantly increased rostral to the syrinx (C2 - C3 pooled, $p < 0.01$ and $p < 0.0001$ respectively). At three weeks, an increase was observed in the cervical cord, both at the site of syrinx (C7 - C8 pooled, $p < 0.05$) and rostral to the syrinx (C2 - C3 pooled, $p < 0.0001$). At six weeks, AQP4 expression had significantly increased in the cervical and thoracic cord at the site of the syrinx (C7-C8, $p < 0.01$ and T1 - T2 pooled, $p < 0.05$) and rostral to the syrinx (C2-C3 pooled, $p < 0.001$).

FIG. 3. Representative images showing (A) colocalization of glial fibrillary acidic protein (GFAP; red) and aquaporin-4 (AQP4; green) in C8 spinal cord of a 1 week sham-injected control rat. A small cavity is present in the gray matter of the right dorsal horn (arrow). (B) Higher magnification view of the cavity showing GFAP-positive astrocytes surrounding the cavity (arrow heads). Higher magnification view of inset in (B) shows (C) colocalization, (D) GFAP expression and (E) AQP4 expression. Scale bar is (A) 500 μ m; (B-E) 50 μ m. Color image is available online at www.liebertpub.com/neu

FIG. 4. Immunolocalization of aquaporin-4 (AQP4; green) and glial fibrillary acidic protein (GFAP; red) in rat spinal cord at 3 days, 1 and 3 weeks following syrinx (*) induction. Animals received four intraparenchymal quisqualic acid injections and a subarachnoid kaolin injection. Left panels show colocalization of GFAP and AQP4 (upper), and GFAP positive astrocytes around the syrinx (lower) indicated by white arrow heads. High magnification view of insets shows colocalization, GFAP and AQP4 expression adjacent to the syrinx (I) and away from the syrinx (II). All sections shown are taken from the region of quisqualic acid injection, between spinal level C7-T1. Scale bar is (left panels) 500 μ m; (high magnification images) 50 μ m. Color image is available online at www.liebertpub.com/neu

A similar result was observed when AQP4 was presented as a ratio with GFAP, with significant increases in AQP4 expression observed in syrinx animals, compared with controls (Fig. 7). At three days, the increase in AQP4 rostral to the syrinx (C2 and C3 pooled) was significant ($p < 0.0001$). At one week, the increase in AQP4 was only significant in the thoracic cord caudal to the syrinx (T6–T7 pooled, $p < 0.05$). At three weeks, the result was dampened, with a significant increase in AQP4 only observed in the upper cervical cord (C2 and C3 pooled, $p < 0.05$). Similarly, at six weeks,

the observed increase in AQP4 expression was less marked and the increase was only significant in the cervical cord at the site of the syrinx (C7–C8 pooled, $p < 0.01$). At 12 weeks, AQP4 expression was still significantly increased, although to a lesser extent, in the upper cervical cord (C2 and C3 pooled, $p < 0.01$).

Western blot analysis of GFAP expression alone yielded a very different result (Fig. 8). At three days, one week, three weeks, and six weeks, there was a decrease in GFAP expression in syrinx animals, compared with controls at all spinal levels investigated. At

FIG. 5. Immunolocalization of aquaporin-4 (AQP4; green) and glial fibrillary acidic protein (GFAP; red) in rat spinal cord at 6 and 12 weeks following syrinx (*) induction. Animals received four intraparenchymal quisqualic acid injections and a subarachnoid kaolin injection. Left panels show colocalization of GFAP and AQP4 (upper), and GFAP positive astrocytes around the syrinx (lower) indicated by white arrow heads. High magnification view of insets shows colocalization, GFAP and AQP4 expression adjacent to the syrinx (I) and away from the syrinx (II). All sections shown are taken from the region of quisqualic acid injection, between spinal level C7-T1. Scale bar is (left panels) 500 μ m; (high magnification images) 50 μ m. Color image is available online at www.liebertpub.com/neu

three days, this decrease was significant rostral to the syrinx (C2 and C3 pooled, $p < 0.05$). At one week following syrinx induction, the decrease in GFAP expression was significant in the thoracic cord, both at the level of syrinx (T1–T2 pooled, $p < 0.001$) and caudal to the syrinx (T6–T7 pooled, $p < 0.0001$). At three weeks, GFAP levels had significantly decreased rostral to the site of syrinx induction (C2 and C3 pooled, $p < 0.01$), at the level of syrinx (C7–C8 pooled, $p < 0.05$), and caudal to the syrinx (T6–T7 pooled, $p < 0.05$). At the six-week time-point, GFAP levels were significantly lower in syrinx animals, compared with controls in the cervical and thoracic cord, away from the syrinx (C2 and C3 pooled, $p < 0.01$ and T6–T7 pooled, $p < 0.01$, respectively). In the 12-week syrinx animals, GFAP levels had increased in the cervical and lower thoracic cord, compared with controls. However, this was not significant.

Discussion

The results from this study demonstrate a significant increase in AQP4 expression at the level of syrinx in an animal model of post-traumatic syringomyelia at three and six weeks following the initial surgery. A significant increase in AQP4 expression also was observed rostral to the syrinx at all time-points except in the acute stage (three days following syrinx induction). Immunoreactivity

demonstrated GFAP-positive astrocytes around the syrinx cavity at all time-points. Astrocytes expressing AQP4 also were observed directly adjacent to the syrinx cavity at all time-points. At six and 12 weeks, AQP4 expression appeared highest directly adjacent to the syrinx cavity, compared with regions away from the syrinx within the same spinal section. The distribution and expression of GFAP demonstrated using immunostaining and western blotting suggests that while overall numbers of GFAP-positive astrocytes decreased (except at the latest time-point, although this was not significant), astrocytes migrated towards the syrinx cavity. The immunostaining indicates that astrocytes directly surrounding the syrinx cavity are expressing AQP4, and at six and 12 weeks AQP4 is most strongly expressed around the syrinx. The increase observed around the syrinx at the six-week time-point correlates with the western blotting results at 12 weeks; however, the western blotting indicates that the overall expression is not significantly higher.

It was previously thought that AQP4 had only two isoforms, M23 (32 kDa) and M1 (34 kDa).²⁵ However, there have been numerous studies using native tissues which have demonstrated multiple AQP4 immunopositive bands,^{23,26,27} and more recently additional AQP4 isoforms have been characterized in the rat brain, including Mz (36 kDa) and 38 kDa protein.^{28–32} A study investigating AQP4 in rat kidney reported two bands at ~ 31 and 52 kDa.

FIG. 6. Ratio of aquaporin-4 (AQP4) expression from western blot analysis at different spinal levels in control and syrinx rats at (A) 3 days, (B) 1 week, (C) 3 weeks, (D) 6 weeks and (E) 12 weeks following syrinx induction. Results are mean $\pm$ standard error of the mean ($n=3$ for control and syrinx animals, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). (F) Representative western blot showing AQP4 (~37kDa) and actin expression in C7 spinal segments taken from control (c) and syrinx (s) animals at the 6-week time-point.

The authors suggested that the higher molecular weight band corresponds to a glycosylated form of AQP4, while the lower molecular weight band denotes the non-glycosylated form.³³ Multiple bands have been reported in studies of other AQPs which have also been attributed to differences in glycosylation in the higher molecular mass forms.^{34,35} Another study found that immunoblotting using an anti-AQP4 antibody gave rise to bands at 31, 34, 59, and 64 kDa. The authors suggested that the higher molecular mass bands may be dimers not N-glycosylated complexes, as digestion with Peptide:N-glycosidaseF did not affect the band size.²⁶ A study demonstrating a 30 and 66 kDa band also suggested that the larger band represented dimeric subunits of the M23 isoform.³⁶ The western blotting results reported in this manuscript demonstrated two robust bands at ~37 kDa and ~45 kDa, and a faint band at ~28 kDa. Based on previous studies it is likely that the lower molecular weight bands observed correspond to AQP4 isoforms, while the ~45 kDa band is likely to be a glycosylated form or a dimer of AQP4. A previous study carried out in our laboratory found that in this excitotoxic model of post-traumatic syringomyelia, the blood-spinal cord barrier (BSCB) is still impaired surrounding a syrinx cavity even at 12 weeks following the initial syrinx induction.¹⁷ In this study, the most significant leakage of tracer across the BSCB was observed at the earlier time-points (three days and one week). It is possible that at three days and one week, AQP4 levels were insufficient to remove fluid that was leaking from the surrounding vasculature. At the later stages, AQP4

may be contributing to astrocytic swelling around the syrinx, rather than eliminating fluid from the leaky blood vessels. Conversely, it may be that AQP4 is simply increasing in an attempt to combat the excess fluid caused by an influx in CSF and not contributing to the fluid accumulation. This theory is supported by a study investigating the role of AQP4 in hydrocephalic edema. Bloch and associates used a well-established kaolin model of hydrocephalus in AQP4-null mice.³⁷ Kaolin injected into the cisterna magna of mice caused ventricular enlargement, increased intracranial pressure and increased brain water content. It was found that in AQP4-null mice, development of hydrocephalus occurred more rapidly than in wild-type mice. It was suggested that removal of excess CSF from the parenchyma occurs primarily through AQP4-dependent pathways.³⁷

In this study, when AQP4 was presented as a ratio of GFAP expression, the trend of increased AQP4 expression in syrinx animals became more prominent in the acute stages (three days and one week), while this trend was less evident in the chronic animals (three-, six-, and 12-week time-points). This suggests that astrocytes may be overexpressing AQP4 in the initial stages of syrinx formation, while in the later stages more astrocytes are present, contributing to the increase in AQP4 expression.

A decrease in astrocytes following spinal cord injury has been previously reported in rats,³⁸ which correlates with the decrease in GFAP we observed. However, in the study by Zai and associates, astrocyte depletion had recovered by six weeks. In our study GFAP expression levels had started to increase after 12 weeks. This

FIG. 7. Ratio of aquaporin-4 (AQP4) expression with glial fibrillary acidic protein (GFAP) expression from western blot analysis. AQP4 and GFAP expression was normalized to Actin. Expression is shown at different spinal levels in control and syrinx rats at (A) 3 days, (B) 1 week, (C) 3 weeks, (D) 6 weeks and (E) 12 weeks following syrinx induction. Results are mean $\pm$ standard error of the mean ($n=3$ for control and syrinx animals, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). (F) Representative western blot showing AQP4 (~37 kDa), GFAP (~51 kDa) and actin expression in C2 spinal segments taken from control (c) and syrinx (s) animals at the 3-week time-point.

suggests that the enlargement of the syrinx continues to cause damage to the spinal tissue or prevents the repopulation of astrocytes. Other studies also have reported an increase in AQP4, following cerebral ischemia³⁹ and in rat spinal cord following an electrical shock.⁴⁰ While, a decrease in AQP4 levels has been reported in a model of brain injury.⁴¹ Nesic and colleagues reported a decrease in AQP4 expression in the acute stages of spinal cord injury and increases in the chronic stages following spinal cord injury in rats.²³ Syringomyelia is generally thought to be a condition caused by alterations in CSF dynamics in the subarachnoid space. In the case of post-traumatic syringomyelia, arachnoiditis occurring after the initial trauma may cause a flow obstruction in the subarachnoid space. There is evidence that CSF does flow into the spinal parenchyma from the subarachnoid space via perivascular spaces.^{42,43} There is also an increase in the flow of CSF into the cord at the level of arachnoiditis.^{11,12} Given this, it is possible that AQP4 is facilitating the removal of fluid from the syrinx, but is unable to keep up with the influx of CSF into the spinal parenchyma. To determine whether AQP4 is removing water from the

syrinx cavity in our animal model, or exacerbating the problem it would be useful to use pharmacological interventions to modulate AQP4 expression. Alternatively, adapting the model of post-traumatic syringomyelia to AQP4 knockout mice would enable a more definitive determination of the role of AQP4.

In this study, we used immunohistochemistry and western blotting to investigate the expression of AQP4 in spinal cords of rats with a noncommunicating extracanalicular syrinx. Previous studies have reported that AQP4 is constitutively expressed in glial cells throughout the spinal cord and in the ependymal cells lining the central canal.⁴⁴ AQP4 labeling is particularly distinct on astrocytic foot processes in close proximity to or in contact with blood vessels.⁴⁴ The results of our study were consistent with these reports, with AQP4 abundantly expressed at the BSCB (surrounding capillaries) and CSF interfaces (at the glia limitans interna and externa). Previous papers have suggested that AQP4 is expressed in protoplasmic astrocytes in the gray matter which have few GFAP immunopositive processes.^{23,45,46} GFAP is typically expressed in fibrous astrocytes.⁴⁵ This is consistent with the lack of

FIG. 8. Ratio of glial fibrillary acidic protein (GFAP) expression from western blot analysis at different spinal levels in control and syring rats at (A) 3 days, (B) 1 week, (C) 3 weeks, (D) 6 weeks and (E) 12 weeks following syring induction. Results are mean $\pm$ standard error of the mean ($n=3$ for control and syring animals, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). (F) Representative western blot showing GFAP (~ 51 kDa) and actin expression in C2 spinal segments taken from control (c) and syring (s) animals at the 3-week time-point.

co-localization between AQP4 and GFAP observed in this study, predominantly in the gray matter. GFAP expression was instead observed in glial processes adjacent to astrocytic endfeet densely labeled with AQP4.

This distribution of AQP4 suggests that it may play a role in regulating fluid transport between the spinal cord and bloodstream and the CSF. This is supported by studies that found a reduction in osmotic swelling in the dorsal horns of spinal cord from AQP4 $^{-/-}$ mice.⁴⁷ Similarly, following spinal cord injury, AQP4 $^{+/+}$ mice have been found to have increased spinal cord swelling compared with AQP4 $^{-/-}$ mice.²² In contrast to this, a decrease in spinal cord edema has been observed when AQP4 expression was enhanced in a mouse model of spinal cord injury.⁴⁸ Recent publications have reported changes in AQP4 expression in rodent brain and spinal cord in association with a number of conditions including trauma.^{19,23} The effects of changes in AQP4 expression vary and can be beneficial or undesirable. For example, in studies measuring brain water swelling following brain injury, AQP4 has been found to facilitate water removal in vasogenic edema; however, it contributes to astrocytic swelling in cytotoxic (cellular) edema.^{21,49}

Given the changes in AQP4 expression patterns observed in this study, it would be of interest to investigate other AQPs, including AQP1 and AQP9, which also are expressed in astrocytes^{40,45,50} to see if they act synergistically with AQP4. We have demonstrated changes in AQP4 expression around post-traumatic syrinxes. Whether these changes play a detrimental or beneficial role in syring formation or enlargement remains to be determined.

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Address correspondence to:

Sarah J. Hemley, PhD

The Australian School of Advanced Medicine

Level 1, 2 Technology Place

Macquarie University

New South Wales 2109 Australia

E-mail: sarah.hemley@mq.edu.au