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INWARDLY RECTIFYING POTASSIUM CHANNEL 4.1 EXPRESSION IN POST-TRAUMATIC SYRINGOMYELIA

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Abstract—Post-traumatic syringomyelia (PTS) is a serious neurological disorder characterized by fluid filled cavities that develop in the spinal cord. PTS is thought to be caused by an imbalance between fluid inflow and outflow in the spinal cord, but the underlying mechanisms are unknown. The ion channel Kir4.1 plays an important role in the uptake of K^+ ions from the extracellular space and release of K^+ ions into the microvasculature, generating an osmotic gradient that drives water movement. Changes in Kir4.1 expression may contribute to disturbances in K^+ homeostasis and subsequently fluid imbalance. Here we investigated whether changes in Kir4.1 protein expression occur in PTS. Western blotting and immunohistochemistry were used to evaluate Kir4.1 and glial fibrillary acidic protein (GFAP) expression in a rodent model of PTS at 3 days, 1, 6 or 12 weeks post-surgery. In Western blotting experiments, Kir4.1 expression increased 1 week post-surgery at the level of the cavity. Immunohistochemical analysis examined changes in the spinal parenchyma directly in contact with the syrinx cavity. In these experiments, there was a significant decrease in Kir4.1 expression in PTS animals compared to controls at 3 days and 6 weeks post-surgery, while an up-regulation of GFAP in PTS animals was observed at 1 and 12 weeks. This suggests that while overall Kir4.1 expression is unchanged at these time-points, there are many astrocytes surrounding the syrinx cavity that are not expressing Kir4.1. The results demonstrate a disturbance in the removal of K^+ ions in tissue surrounding a post-traumatic syrinx cavity. It is possible this contributes to water accumulation in the injured spinal cord leading to syrinx formation or exacerbation of the underlying pathology.
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Key words: Kir4.1, astrocyte, syrinx, post-traumatic syringomyelia.

Post-traumatic syringomyelia (PTS) is a serious complication of spinal cord trauma, occurring in approximately 28% of spinal cord-injured patients (Milhorat et al., 1995; Schaller et al., 1999; Brodbelt and Stoodley, 2003; Brodbelt et al., 2003b; Carroll and Brackenridge, 2005; Bonfield et al., 2010; Wong et al., 2012). PTS is characterized by the formation of high-pressure fluid-filled cysts within the spinal cord. As a syrinx enlarges, it damages the surrounding spinal cord tissue, resulting in pain, paralysis and loss of sensation (Milhorat et al., 1995; Bogdanov and Mendelevich, 2002). Long-term results from surgical treatment are poor, and reoperation is often necessary (Karam et al., 2014). To develop improved treatment strategies it is critical to have a better understanding of the mechanisms underlying syrinx formation and enlargement.

Syrinx formation and enlargement have been assumed to be related to abnormalities in cerebrospinal fluid (CSF) hydrodynamics (Bilston et al., 2010; Cheng et al., 2012). According to these theories arachnoiditis or inflammation surrounding the spinal cord obstructs the normal flow of CSF, causing pressure changes in the subarachnoid space that are thought to drive fluid into the spinal cord (Oldfield et al., 1994; Heiss et al., 1999; Brodbelt et al., 2003a,b). Since increasing pressure on the outside of the spinal cord cannot create an expanding cyst within the cord (Hall et al., 1980; Klekamp et al., 2001; Brodbelt et al., 2003a,b), there may be other sources of fluid. It is likely that the pathogenesis of PTS is complex and molecular and cellular processes also contribute to the formation of a syrinx following spinal cord injury.

Ion channels, such as the inwardly rectifying potassium channel 4.1 (Kir4.1) are thought to perform an important role in maintaining cell volume in the brain and spinal cord. Kir4.1 is expressed in glial cells within the central nervous system, predominantly in astrocytes. Kir channels maintain K^+ ion homeostasis, by facilitating the movement of K^+ from the extracellular

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Abbreviations: AQP4, aquaporin 4; CSF, cerebrospinal fluid; DTT, dithiothreitol; EGTA, ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GFAP, glial fibrillary acidic protein; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; KCl, potassium chloride; Kir4.1, inwardly rectifying potassium channel 4.1; NDS, normal donkey serum; PMSF, phenylmethylsulfonyl fluoride; PTS, post-traumatic syringomyelia; TPBS, tris phosphate-buffered saline.

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space into glial cells and removal to areas of low K^+ concentration such as CSF and blood. A disruption in K^+ transport may prevent the clearance of intracellular K^+, and this in turn can lead to cytotoxic edema by an influx of water from the vasculature into astrocytes via aquaporin 4 (AQP4) water channels (Nagelhus et al., 2004; Pannicke et al., 2004). Kir4.1 has been found to be down-regulated in a number of pathological conditions, including amyotrophic lateral sclerosis (Bataveljic et al., 2012), Alzheimer's disease (Wilcock et al., 2009), spinal cord injury, and in the presence of glial scarring (MacFarlane and Sontheimer, 1997; Bordey et al., 2001). These findings point to the need for further research into Kir4.1 and its effect on conditions that involve the accumulation of fluid in the central nervous system. The aim of this study was to characterize Kir4.1 expression in a rodent model of PTS.

EXPERIMENTAL PROCEDURES

Following approval from the Animal Care and Ethics Committee of Macquarie University, 61 male Sprague–Dawley rats weighing 297 ± 93 g (mean ± SD), age range 4–7 weeks were divided into two experimental groups: 24 animals for Western blotting (Table 1) and 37 animals for immunohistochemistry (Table 2). Western blotting and immunohistochemistry techniques were used to investigate Kir4.1 and GFAP expression in control (laminectomy-only) and syrinx animals (see details below). In Western blotting experiments six control (laminectomy-only) and 18 syrinx animals were divided into three time-point groups (1, 6 and 12 weeks post-surgery) (Table 1). For immunohistochemical analysis eight control animals and 29 syrinx animals were divided into four time-points (3 days, 1, 6 and 12 weeks post-surgery) (Table 2). The rate of cavity formation was calculated from spinal cord sections used for immunohistochemical analysis.

Syrinx induction

The post-traumatic model of syringomyelia was created using a controlled pneumatic compression device (Infinite Horizon Impactor, Precision Systems and Instrumentation, LLC, Kentucky, USA) to produce the initial spinal cord cyst, followed by a subarachnoid injection of kaolin as previously described (Wong et al., 2015).

All procedures were performed in a sterile field under general anesthesia induced with 5% isoflurane in oxygen (1 L/min) and maintained with 2–2.5% isoflurane through a nose cone. Animals were placed prone, and the skin was shaved and prepared with povidone iodine. An incision was made over the cervicothoracic junction and C7–T1 laminectomies were performed. The spinous processes of C6 and T2 were clamped and stabilized using a stereotactic device. The spinal cord impactor was positioned over the cord with the 2-mm impactor tip just touching the dorsal surface. The spinal cord impactor was calibrated to deliver 75 kDyn to the spinal cord. Previous research in our laboratory demonstrated that 75 kDyn is the ideal force to produce PTS without producing any significant paralysis or neurological deficits in animals (Wong et al., 2015). A 5 μL suspension of 250 mg/mL kaolin (Sigma–Aldrich, St. Louis, Missouri, USA) in sterile saline was then injected into the subarachnoid space. The wound was closed with 4-0 Absorbable Coated Vicryl sutures (Ethicon, Johnson & Johnson Medical Pacific Pty Ltd, Sydney, Australia). At the conclusion of the operation 0.05 mg/kg of 324 μg/mL buprenorphine in 5% glucose solution was administered subcutaneously. Subsequent doses were given as needed. The animals were allowed food and water ad libitum and monitored for any signs of excessive weight loss, limb weakness, or excessive self-grooming.

Table 1. Experimental groups for Western blotting: surgical procedure and survival time in experimental rats

Experimental group	Initial operation	No. of animals at each survival point		
		1 week	6 weeks	12 weeks
Control	Laminectomy	2	2	2
Syrinx induction	Laminectomy + spinal cord injury + kaolin injection	6	6	6

Western blotting

At 1, 6 or 12 weeks after the syrinx induction surgery, animals were anesthetized with 5% isoflurane in 1 mL/min oxygen and perfused by intracardiac injection of 2000 IU heparin in 400 mL ice-cold phosphate-buffered saline. Spinal segments; C2–3, C5–6, C7–8 and T1–2 were snap frozen on dry ice.

The spinal tissue was placed in ice cold lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, California, USA). The tissue was homogenized using a FastPrep®-24 tissue and cell homogenizer (MP Biomedicals, Solon, Ohio, USA) for 20 s at 4.0 m/s. After vortexing for 5 s 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, USA).

Samples containing 30 μg of protein were heated for 10 min at 70 °C with an appropriate volume of 4× NuPAGE® LDS sample buffer, 10× NuPAGE® Reducing Agent (Invitrogen, Carlsbad, California, USA) and up to 6.5 μL of deionized water for a total volume of 10 μL. Samples were loaded onto a NuPAGE® Novex 10% 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 4% (w/vol) non-fat dry milk in tris-buffered saline (TBS) with Tween-20 for 60 min at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies: anti-Kir4.1 (1:800, Alomone Labs, Jerusalem, Israel, APC-035), anti-GFAP (1:60 000, Merck Millipore, Darmstadt, Germany,

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