

## Aquaporin-4 and spinal cord injury

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### Abstract

Edema formation is a major problem following traumatic spinal cord injury (SCI) that acts to exacerbate secondary damage. Severity of edema correlates with

reduced neurological outcome in human patients. To date, there are no effective treatments to directly resolve edema within the spinal cord. The aquaporin-4 (AQP4) water channel is found on membranes of astrocytic endfeet in direct contact with blood vessels, the glia limitans in contact with the cerebrospinal fluid and ependyma around the central canal. Being so locally expressed at the interface between fluid and tissue allow AQP4 channels to play an important role in the bidirectional regulation of water homeostasis under normal conditions and following trauma. With the need to better understand the pathophysiology underlying the devastating cellular events in SCI, animal models have become an integral part of exploration. Inevitably, several injury models have been developed (contusion, compression, transection) resulting in difficult interpretation between studies with conflicting results. This is true in the case of understanding the role of AQP4 in the progression and resolution of edema following SCI, whose role is still not completely understood and is highly dependent on the type of edema present (vasogenic vs cytotoxic). Here, we discuss regulation of AQP4 in varying injury models and the effects of potential therapeutic interventions on expression, edema formation and functional recovery. Better understanding of the precise role of AQP4 following a wide range of injuries will help to understand optimal treatment timing following human SCI for prime therapeutic benefit and enhanced neurological outcome.

**Key words:** Spinal cord injury; Astrocyte; Aquaporin-4; Edema; Water channel

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**Core tip:** Edema formation is a major problem following spinal cord injury (SCI) that acts to exacerbate secondary damage. Animal models have become an integral part of understanding this pathophysiology. Several SCI models have been developed, resulting in difficult interpretation between studies with conflicting results. This is true for the role of aquaporin-4 (AQP4) water

channels in the development and resolution of edema following SCI. Here, we discuss regulation of AQP4 in varying models and the effect of current interventions on expression, edema formation and functional recovery. Understanding the precise role of AQP4 will help to determine optimal treatments following human SCI.

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## SPINAL CORD INJURY

Spinal cord injury (SCI) is a debilitating and life altering condition. Given the current lack of therapeutic treatments available, the irreversible loss of motor and sensory function often results in reduced quality of life and increased morbidity rates<sup>[1]</sup>. The initial physical insult to the spinal cord (primary injury) is followed by a cascade of secondary cellular events that act to exacerbate the initial detriment (secondary injury)<sup>[2]</sup>. The progressive damage includes break down of the blood-spinal cord barrier (BSCB)<sup>[3]</sup>, formation of a glial scar and cavitation at the injury site<sup>[4]</sup>, accumulation of edema, hemorrhage, inflammation and excitotoxicity and eventual cell death<sup>[2]</sup>. The majority of current SCI research is aimed at better understanding these secondary processes. Reasoning states that, while the damage due to the initial injury is unpreventable, the resulting secondary damage may be averted or even reversed to allow for better neurological outcome in patients suffering from dysfunction.

With the need to understand the pathophysiology underlying the devastating cellular events in SCI, animal models have become an integral part of exploration. To date, several injury models have been utilized. There are currently three main types of injury used in SCI research: Contusion, compression and transection<sup>[5]</sup>. Contusion injuries are produced by striking the bare spinal cord with a probe delivered at a determined force<sup>[6-8]</sup> or with a weight drop system<sup>[9]</sup>. Compression injuries are created by constricting the exposed cord with a clip<sup>[10]</sup>, forceps<sup>[11]</sup> or application of a weight<sup>[12]</sup> for sustained periods of time. Finally, transection models are performed by opening the dura and using a blade to partially or completely cut the spinal cord<sup>[13]</sup>. Contusion and compression models are thought to more accurately represent human injury compared to transection models<sup>[5,13,14]</sup>. For further detail on animal models of SCI, see reviews by Rosenzweig *et al*<sup>[5]</sup>, Kwon *et al*<sup>[13]</sup> and Cheriyan *et al*<sup>[15]</sup>.

With the advent of varying injury models with equally variable parameters, comes the inevitable difficulty of interpreting results between studies examining similar topics and interventions. This is true in the

case of understanding the role of aquaporin-4 (AQP4) water channels in the progression and resolution of edema following SCI. Thus, the purpose of this review is to clearly delineate injury models and paradigms, therapeutic treatments, AQP4 expression levels and functional outcomes.

## RESEARCH

A thorough literature search was conducted using the PubMed library for "aquaporin-4," "spinal cord injury" and "edema". A total of 18 studies focused on AQP4 expression and modification following SCI were included in this review. Of these studies, four were AQP4 expression studies, eleven were AQP4 modulation studies and three were AQP4 null studies. Of the expression studies, two were done in contusion models of SCI and one was done in a compression model. One of the contusion studies also included post mortem analysis of human tissue. Of the modulation studies, six were done in contusion models and the remaining five were done in compression models of injury. Finally, of the three AQP4 null studies found, one was completed in a contusion model, another was completed in a compression model and the last was done in a transection model. We have organized this review according to injury model.

## SPINAL CORD EDEMA

Edema occurs during the acute phase after injury and spreads rostral and caudal within 24 to 48 h leading to increased cord volume over time<sup>[16]</sup>. Edema can be due to vasogenic or cytotoxic mechanisms, or a combination of the two. Vasogenic edema results from breakdown of the BSCB and accumulation of fluid into the extracellular space, while cytotoxic edema is characterized by fluid flow into astrocytes resulting in cellular swelling. Injury severity correlates well with the development and alleviation of edema at the injury site<sup>[17,18]</sup>. The extend of edema can have a large effect on the resulting secondary damage and neurological outcome following injury to the central nervous system (CNS)<sup>[16,19-21]</sup> and persists longer than hemorrhage<sup>[17]</sup>. Patients displaying single level edema show greater ASIA score recovery compared to those with diffuse or multilevel edema<sup>[21,22]</sup>. Interestingly, some studies show that age is inversely correlated with length of edema and that women show a trend towards greater edema levels<sup>[17]</sup>, while others show no correlation<sup>[19]</sup>. This effect, to our knowledge, has not been tested in animal models of SCI.

In addition to edema, degree of neurological outcome and recovery from SCI are also correlated with hemorrhage<sup>[21,22]</sup>, amount of cord and central canal compression and extent of soft tissue injury<sup>[21]</sup>. SCI in humans and rodent models has been shown to lead to compression of the cord tissue against the surrounding dura leading to elevated cord pressure, reduced blood supply and eventual tissue death<sup>[2,23,24]</sup>. Vascular

disruption and break down of the BSCB contribute to increased fluid, protein and blood accumulation within the spinal cord<sup>[2,3,25]</sup>. BSCB permeability was found to be biphasic, with increased leakage occurring 24 h after injury, then again between 3 and 7 d in a rodent contusion models<sup>[3]</sup>. Repair of the barrier is evident by 2 to 3 wk post-trauma<sup>[3]</sup>, but disruption may last for longer periods of time depending on the injury severity<sup>[25]</sup>.

Current surgical interventions, such as decompression and stabilization, do not reduce edema and cord pressure at the injury site or reduce necrosis<sup>[23]</sup>. In addition, the usefulness of decompression on varying types of SCI and timing post injury remains controversial<sup>[26]</sup>. Following clinical trial, methylprednisolone (MP) became the only treatment used immediately after SCI<sup>[27]</sup> to reduce ischemia<sup>[28]</sup> and decrease edema and peroxidase effects on cell membranes<sup>[29,30]</sup>. It is noteworthy, however, that the Food and Drug Administration (FDA) has not formally approved the use of MP following SCI. In addition, because of the uncertainty of its beneficial and/or harmful effects, MP remains highly controversial<sup>[31]</sup> and its use is waning<sup>[23]</sup>. As more is being uncovered about the mechanisms underlying edema formation following injury to the CNS, interventions aimed at this issue are becoming more clinically important. To date, there is no widely accepted and effective treatment for edema following SCI.

## AQP4 IN THE SPINAL CORD

The movement of water across membranes is facilitated by the aquaporin (AQP) family of membrane-bound water channels<sup>[32,33]</sup>. AQP4 is the most abundantly expressed water channel in both the gray and white matter of the cervical, thoracic and lumbar segments of the spinal cord<sup>[23,34-36]</sup>. AQP4 is localized on the membranes of astrocytic endfeet in direct contact with vasculature, at the glia limitans in contact with the cerebrospinal fluid and the ependyma around the central canal<sup>[34,37-40]</sup>. Expression is also found to be equal at both symmetric (inhibitory) and asymmetric (excitatory) synapses<sup>[35]</sup> and in contact with neuronal cell bodies<sup>[39]</sup>. AQP4 channels are formed by six transmembrane helix proteins and arrange themselves as a tetramer in the astrocytic membrane<sup>[41]</sup>. Each monomer functions as its own pore for water transport<sup>[41]</sup>. Groups of tetramers form clusters known as orthogonal array particles<sup>[38,39,42-44]</sup>, a feature that is unique to AQP4<sup>[45]</sup>. This grouping of AQP4 channels is thought to allow for more effective anchoring of the proteins in the membrane compared to monomers or tetramers alone<sup>[40]</sup>. Being so locally expressed at the interface between fluid and tissue allows AQP4 channels to play a central function in the bidirectional regulation of water homeostasis under normal conditions and following trauma to the CNS<sup>[23,32,34,37,39,41,46]</sup>. AQP1 and AQP9 are also found in the spinal cord<sup>[34]</sup> although their roles following SCI are not discussed in this review.

## AQP4 EXPRESSION FOLLOWING HUMAN SCI

In a study by Nesic *et al.*<sup>[47]</sup>, three human cord samples were obtained from the Guest *et al.*<sup>[48]</sup> study on SCI-induced demyelination and analyzed for expression of AQP4. All tissue used in this study was obtained from individuals that had been injured for a duration of 1 to 2 years. Interestingly, it was found that, following cervical injury, all cord samples showed glial fibrillary acidic protein (GFAP)-labeled astrocytes both with and without AQP4 labeling at the lesion epicenter<sup>[47]</sup>. Typically, AQP4 is found on all GFAP-positive astrocytes, while some distant arborizations show AQP4 labeling alone without GFAP<sup>[36]</sup>. This was the first report of this reversed occurrence. In contrast to the lack of expression on astrocytes at the lesion epicenter and around cyst formations, AQP4 labeling was increased in the spared white matter compared to uninjured cord tissue<sup>[47]</sup>. To our knowledge, this is the only study to examine AQP4 expression in human tissue samples.

## AQP4 EXPRESSION FOLLOWING CONTUSION SCI

We will first explore the results of AQP4 expression studied in contusion models of SCI. In the chronically injured human spinal cord, Nesic *et al.*<sup>[47]</sup> showed marked upregulation of AQP4 in spared white matter, as well as GFAP-positive astrocytes lacking AQP4 expression at the lesion epicenter. The same research group then performed an extensive characterization of AQP4 expression following contusion injury in a rat model. In contrast to their human tissue findings, rat tissue showed GFAP-labeled astrocytes lacking AQP4 during only the acute and subacute periods following injury (up to 14 d) and increased AQP4 expression in both the grey and white matter in the chronic setting (at day 21 to 11 mo post injury)<sup>[36,47]</sup>. This trend, however, was dependent on injury severity, where more severe injuries showed longer durations of downregulated AQP4<sup>[47]</sup>. To support this, Western blot analysis showed decreased levels of AQP4 protein within the first weeks post injury and consistently elevated levels from 2 wk to 9 mo post SCI<sup>[36]</sup>. GFAP levels began to increase as early as 12 h post injury and continued to increase through 9 mo<sup>[36]</sup>. The role of chronically increased AQP4 levels still remains to be determined. From these results, it was hypothesized that astrocytes lacking AQP4 at the lesion site may represent newly migrated cells<sup>[36,47]</sup>, which have been shown to proliferate soon after contusive SCI, along with oligodendrocytes<sup>[49,50]</sup>. This is interesting considering AQP4's perceived involvement in astrocyte migration. Observation of astrocytes isolated from AQP4 null mice showed slower migration compared to those isolated from wildtype controls in an *in vitro* scratch test<sup>[39,51]</sup>, therefore migration of AQP4-negative astrocytes following injury must be controlled by other

factors. Nesic *et al.*<sup>[47]</sup> suggest a progression of AQP4-negative astrocytes to AQP4-overexpressing astrocytes as the natural progression post injury, rat astrocytes undergoing the transformation more rapidly than human. Analysis of human tissue post 2 years after injury would be needed to verify this hypothesis.

Interestingly, the same study grouped rat outcome based on injury severity (mild, moderate and severe) and performed analysis of AQP4 protein expression. It was found that at 3 wk post injury, animals showing severe impairment on the Basso, Beattie, Bresnahan (BBB) locomotor scale<sup>[52]</sup> had lower levels of AQP4 protein<sup>[47]</sup>. Lower levels of AQP4 appeared to be correlated with reduced motor recovery following severe injury in this contusion model of SCI, which differs from the concept of the detrimental effects of AQP4 overexpression after injury<sup>[47]</sup>. Nesic *et al.*<sup>[36,47]</sup> also showed increased tissue water content by 3 d at the lesion site and at distal rostral and caudal segments at 2 and 5 mo. These results present the possibility that, although its levels increase, changes in localization of AQP4 (and possibly its anchoring proteins) may lead to impaired water clearance (and cytotoxic edema) in the chronically injured cord<sup>[36]</sup>. Conversely, decreased AQP4 levels early after injury would result in increased vasogenic edema<sup>[36]</sup>. Comparing back to the previously mentioned human MRI studies, edema severity correlates well with neurological outcome, making for a reasonable assessment.

Rodent T2 MRI images revealed a hyperintense signal at and around the injury site beginning at 7 d post contusion injury<sup>[47]</sup>. By 2 wk post injury, there is the development of defined fluid filled cavities at the lesion site<sup>[47]</sup>. This time line correlated well with the initial down regulation of AQP4 (leading to vasogenic edema) followed by increases in AQP4 expression<sup>[47]</sup>. Although no evidence has been made to directly link the two events, the authors claim that increases in AQP4 do not appear to influence the formation of cysts within the spinal cord, nor determine the size or enlargement of the cavity<sup>[47]</sup>. This fact will be assessed in a later study.

Chronic pain is a serious complication that patients must cope with following SCI. Interestingly, there has been a correlative link between increased AQP4 expression following contusion injury and development of chronic neuropathic pain as far as 5 mo after injury<sup>[53]</sup>. Peripheral nerve injury has also been shown to lead to increased AQP4 expression within the spinal cord and decreased pain withdrawal thresholds<sup>[54]</sup>. The correlation between these events may be the excessive release of the excitatory neurotransmitter glutamate that has been shown to extend distally from the lesion epicenter as far as 5 mm<sup>[55]</sup> and the observed downregulation of both glutamate transporter 1 and glutamate aspartate transporter following injury<sup>[56]</sup>. Glutamate release due to astrocytic swelling could be responsible for hyperexcitability, resulting in excitotoxicity and eventual neuronal death<sup>[57,58]</sup>. If this were the case, intense mechanical allodynia and hypersensitivity in rodents,

like chronic pain seen in humans, could be attributed to exaggerated and sensitized responses of sensory neurons within the spinal cord<sup>[47,59]</sup>.

Hypoxia is an important factor, which has been correlated with vascular leakage, break down of the BSCB and formation of edema following SCI. Translation of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is upregulated under hypoxic conditions, including SCI beginning at 6 h after injury and reaching its peak at around 3 d<sup>[60]</sup>. Studies have shown a correlation between the upregulation of HIF-1 $\alpha$  and increases in AQP4 expression in SCI<sup>[47]</sup>. The antioxidant melatonin easily crosses the BSCB and has been shown to protect against its disruption following SCI<sup>[61]</sup>. In a mouse contusion model, melatonin was shown to reduce BSCB permeability, reduce cell apoptosis and preserved tight junction proteins<sup>[61]</sup>. In addition, the same study found that spinal cord water content was increased in injured animals, but decreased in melatonin treated animals along with levels of AQP4 protein when examined 48 h after injury<sup>[61]</sup>. Markers of hypoxia (HIF-1 $\alpha$ ) and BSCB permeability (vascular endothelial growth factor, VEGF) were also downregulated with treatment<sup>[61]</sup>. Other studies have also found functional recovery following administration of melatonin<sup>[62,63]</sup> thought to be due to increased pericytes on microvessels and increased BSCB stability<sup>[63]</sup>. In addition, melatonin administration was shown to reduce levels of inducible nitric oxide synthase (iNOS) activity<sup>[62]</sup>. This decreased nitric oxide (NO) production would effectively prevent its actions as a free radical in the exacerbation of secondary injury following SCI<sup>[62]</sup>. Similar studies have examined the downregulation of iNOS activity with L-NNA following SCI and showed that pretreatment with an iNOS inhibitor lead to decreased cord swelling, hemorrhage and edema, decreased cord water content and reduced BSCB permeability<sup>[64]</sup>. In all, this points to the ability of melatonin to induce neuroprotection by combating hypoxia and free radicals, limiting BSCB leakage and mediating edema clearance in contusion injury models.

Possibly by similar mechanisms the free radical scavenger, edaravone, readily crosses the BSCB, and has been shown to reduce infarct and improve neurological outcome in a model of cerebral ischemia<sup>[65]</sup>. This study also showed reduced AQP4 protein expression levels along with decreased levels of edema<sup>[65]</sup>. To our knowledge, this application has yet to be tested to reduce SCI-induced edema. Other antioxidants, such as acetazolamide, have been shown to decrease water permeability through AQP4 in a concentration dependent manner in liposomes<sup>[66]</sup>. Although further research must be done to examine their efficacy following SCI, it shows a promising ability to perhaps reversibly bind AQP4. If the mechanism by which acetazolamide binds to AQP4 is uncovered, it could lead to the development of therapeutic treatments aimed to alter AQP4 function as a means of neuroprotection<sup>[66]</sup>.

Recently, a surgical intervention following contusion SCI has shown an effect on AQP4 expression and



improved functional recovery<sup>[24]</sup>. As mentioned in the introduction, decompressive procedures following SCI do not fully remove the pressure caused by the swollen cord tissue against the surrounding dura matter<sup>[23,24]</sup>. Unlike surgical durotomy, myelotomy allows means to directly remove hemorrhagic and necrotic tissue from the injury site by making a longitudinal incision through the dura and into the spinal cord tissue<sup>[24]</sup>. In addition, it is thought to both reduce and prevent the accumulation of fluid and cavity formation within the spinal cord typical of both human and rat SCI<sup>[67]</sup>. Interestingly, myelotomy performed 24 h after contusion SCI acted to inhibit the upregulation of AQP4 and reduce spinal cord edema for 6 d after injury, and improve functional outcome as far as 6 wk<sup>[24]</sup>. The mechanism by which this surgical intervention alters AQP4 expression is yet to be elucidated.

In our research regarding contusion models, all of the aforementioned studies recorded increased levels of AQP4 at the RNA, protein and histological levels. Those that sought out an intervention found improved outcome through reduction of AQP4 expression<sup>[24,63,68,69]</sup>. Two studies reported the same increase in AQP4, but found improved functional outcome through a further increase in AQP4 expression. In the first of these studies, the estrogen receptor modulator, tamoxifen (TMX), was shown to have beneficial effects if administered during the acute period following a contusion model of SCI<sup>[70-72]</sup> but not during delayed administration<sup>[72]</sup>. Tissue was shown to have increased levels of AQP4 protein and decreased GFAP protein levels 35 d after thoracic contusion<sup>[72]</sup>. Histological analysis showed that cavity formations in animals treated with TMX were smaller, more rounded and had AQP4-heavy expressing borders compared to vehicle treated animals<sup>[72]</sup>. This is an interesting observation as increased AQP4 levels were previously reported to play no role in facilitating the formation of fluid-filled cavities within the cord, nor determine cavity size<sup>[47]</sup>. It is therefore possible that levels of AQP4 that are elevated following TMX administration compared to SCI alone may, in fact, play a role in determining cavity size and enlargement. In correlation with this study, TMX has been shown to decrease BSCB permeability and spinal cord water content after injury, while reducing neuronal death and loss of myelin<sup>[70]</sup>. These are interesting findings as the FDA currently approves of TMX for use in breast cancer treatment. That being said, the mechanisms by which TMX affects AQP4 levels are unknown, and whether its effects are estrogen receptor mediated or not<sup>[72]</sup>. Possibilities of TMX action include: Inhibition of excitatory amino acids (glutamate) release, inhibition of iNOS activity, removal of reactive oxygen species during hypoxia<sup>[70]</sup> and/or by downregulation of pro-inflammatory cytokine production<sup>[71]</sup>.

In the second study, Vitellaro-Zuccarello *et al.*<sup>[73]</sup> showed that following contusion injury, treatment with recombinant human erythropoietin (rhEPO) lead to increased AQP4 immunoreactivity compared to saline

treated animals in both the grey and white matter at 7 and 28 d after injury, but did not alter levels of dystrophin and syntrophin responsible for AQP4 anchoring. Other results included reduced GFAP and chondroitin sulfate proteoglycans labeling compared to control animals<sup>[73]</sup>. In the same study, treated animals showed improved locomotor outcome thought to result from the improved clearance of water from the extracellular space resulting in reduced pressure, which was not directly measured. Mechanisms may include tissue re-oxygenation, leading to AQP4 upregulation<sup>[73]</sup>. In another set of studies, the cytokine rhEPO greatly reduced glial scar and cavity formation and decreased recruitment of inflammatory cells to the injury site when administered within a narrow 30-min window after injury<sup>[74]</sup>. In addition, pro-inflammatory cytokine concentrations were also reduced in treated animals<sup>[74]</sup>. In all, there was an improved functional outcome in the BBB locomotor task<sup>[74,75]</sup> and forced swim test<sup>[75]</sup>. Interestingly, the same study did not find any benefit to locomotion with administration of MP (in agreement with our earlier discussion). Co-administration of the two actually abolished any functional improvements, perhaps by suppressing EPO-mediated upregulation of neurotrophic factors<sup>[74]</sup>. Administration has also been shown to improve spared white matter following contusion injury<sup>[76]</sup>. A recent clinical trial<sup>[77]</sup> showed that administration of erythropoietin (EPO) resulted in reduction of at least one ASIA score (A to B) by 90 d after administration in 3 of 11 patients, in comparison to MP treated patients that showed no improvements in ASIA score by 90 d (8 patients). Notably, there were no health concerns following EPO administration<sup>[77]</sup>. A second clinical trial is being conducted in Canada, although the results of this study are unknown at this time. Mechanisms by which EPO is beneficial post SCI include its role following hypoxia. As we have mentioned, hypoxia leads to expression of HIF-1 $\alpha$ <sup>[47,60]</sup>. Interestingly, the expression of EPO is highly regulated by HIF-1 $\alpha$  and may act to attenuate the negative effects of hypoxia by increasing oxygenation<sup>[78]</sup>. Although the exact neuro-protective mechanisms employed by EPO are not fully understood, it is thought to inhibit apoptosis, modulate synthesis of NO, protect from excess glutamate and restore vascularization and permeability of the BSCB following injury (see review by Carelli *et al.*<sup>[78]</sup>).

As we have seen, there is great variation within the outcomes in contusion models of SCI (Tables 1 and 2). All the studies reported agree that SCI results in chronically increased levels of AQP4. The studies that found decreased levels in AQP4 as beneficial to recovery used the Infinite Horizons Impactor (IH Impactor) in mice<sup>[63]</sup>, the New York University weight drop device (NYU device) in rats<sup>[24]</sup>, a striking bar in rats<sup>[69]</sup> and a weight drop in mice<sup>[61]</sup>. Studies that concluded that a further increase in AQP4 expression was beneficial used the IH Impactor<sup>[72]</sup> or the UTS Impactor<sup>[73]</sup> in rats. Possible differences may simply be due to timing of AQP4 expression analysis. In the 4 studies that reported beneficial effects

**Table 1** Studies of aquaporin-4 expression following spinal cord injury

Ref.	Species	Model	Device	Parameters	Level	AQP4 RNA	AQP4 protein	AQP4 IHC	Water content	Behavioral outcome
Nesic <i>et al</i> <sup>[53]</sup>	Sprague dawley male rats	Contusion	IH Impactor	200 kdyn	T10	↑ AQP4 at 28 d post SCI	↑ AQP4 at 28 d post SCI	↑ AQP4 at 28 d and 5 m post SCI		↑ CNP in animals with increased AQP4
Nesic <i>et al</i> <sup>[56]</sup>	Sprague dawley male rats	Contusion	IH Impactor	150 kdyn, 1 s dwell	T13		↓ AQP4 at 12 h, 24 h, 3 d and 7 d post SCI ↑ AQP4 at 14 d, 35 d, 40 d, 56 d, 3.5 m and 9 m post SCI	↑ AQP4 at 5 m post SCI Astrocytes lacking AQP4 at dpi 14	↑ levels at lesion site at 3 d ↑ levels at all levels at 2 and 5 mo	
Nesic <i>et al</i> <sup>[47]</sup>	Human				C5/6, C2, C8			↑ AQP4 in spared white matter ↓ AQP4 in lesion epicenter (1-2 yr post SCI)		
	Sprague dawley male rats	Contusion	IH Impactor	150 kdyn, 1 s dwell	T10		↑ AQP4 at 2 and 11 m post SCI ↓ AQP4 in severe SCI at 21 d ↓ AQP4 in moderate SCI at 1, 7 and 14 d no change in moderate/mild SCI at 21 d	↑ AQP4-/GFAP+ astrocytes at 14 d post SCI ↑ AQP4 labeling in AQP4+ astrocytes post SCI ↑ AQP4 at 1, 2, 3 and 5 m post SCI	↑ levels at 3 d, 2 and 5 m	BBB outcome coorelated with severity of injury
Leonard <i>et al</i> <sup>[79]</sup>	Rabbits	Compression	Balloon compression	8 atm pressure, 5 min hold	T10			↑ AQP4 at 5 h, 24 h, 3 d and 14 d post SCI	↑ levels at 5 h, 24 h and 3 d	↓ Tarlov score and sensory pin prick in SCI animals

AQP4: Aquaporin-4; IH: Infinite Horizons; BBB: Basso, Beattie, Bresnahan; CNP: Chronic neuropathic pain; GFAP: Glial fibrillary acidic protein; SCI: Spinal cord injury.

following decreased AQP4, measurements were taken between 2 and 7 d post injury. On the other hand, those that reported increases in AQP4 to be beneficial looked at expression from 7 to 35 d. Observation of more chronic and more acute expression analysis in each of the groups, respectively, could help to further understand the differences in each of the studies. Another explanation may be injury severity, as Nesic *et al*<sup>[47]</sup> showed different expression profiles between mild, moderate and severe contusion injuries.

## AQP4 EXPRESSION FOLLOWING COMPRESSION SCI

Compression injuries are another widely used model in SCI studies regarding AQP4 and edema formation. Unlike the Nesic *et al*<sup>[47]</sup> expression paper, there has

been no in depth characterization of AQP4 RNA or protein profiles after compression SCI. Histological assessments in a rabbit model of balloon compression reveal increased AQP4 expression beginning at 5 h post injury and continuing into the chronic setting (14 d post SCI)<sup>[79]</sup>. This was the only study to examine AQP4 expression beyond 3 d post injury.

We have previously mentioned in our discussion of contusion injuries that hypoxia is linked to vascular leakage and the formation of edema following SCI. Along those lines, increased levels of HIF-1 $\alpha$ <sup>[47,60]</sup> and VEGF<sup>[61]</sup> are hallmarks of hypoxia and increased BSCB permeability, respectively. The upregulation of each leads to upregulation of AQP4 following SCI<sup>[47]</sup>. In agreement with the results obtained from melatonin studies in a contusion model<sup>[61,63]</sup>, melatonin following a rat compression model of SCI lead to reduced AQP4

Table 2 Studies utilizing therapeutic interventions to alter aquaporin-4 expression after spinal cord injury

Ref.	Species	Model	Device	Parameters	Level	Treatment	Timing/ duration	AQP4 RNA	AQP4 protein	AQP4 IHC	Water content	Behavioral outcome
Vitellaro-Zuccarello <i>et al</i> <sup>[23]</sup>	Sprague dawley male rats	Contusion	UTS Impactor	1 N, 1 s dwell	T9	rhEPO, 5000 UI/kg	Once, 30 min post injury			↑ AQP4 in untreated at 7 d and 28 d ↑ AQP4 in rhEPO at 7 d and 28 d		↑ in rhEPO treated (BBB)
Guptarak <i>et al</i> <sup>[72]</sup>	Sprague dawley male rats	Contusion	IH Impactor	150 kdyn, 1 s dwell	T10	TMX, 1 mg	Once/day, 14 or 28 d post injury (acute), days 42-56 post injury (delayed)		↑ AQP4 in untreated and 35 d ↑ AQP4 in TMX treated at 35 d	↑ AQP4 in untreated and 35 d ↑ AQP4 in TMX treated at 35 d		↑ in TMX treated at 14 d and 28 d (acute treatment) no BBB recovery in animals treated from day 42-56 (delayed treatment)
Wu <i>et al</i> <sup>[61]</sup>	C57B/16 male mice	Contusion	Weight drop	5 g weight, 10 mm drop	T10	Melatonin, 50 mg/kg	Once, post injury		↑ AQP4 in untreated at 48 h ↓ AQP4 in melatonin treated at 48 h	↑ AQP4 in untreated at 48 h ↓ levels in melatonin treated at 48 h		↑ levels in untreated at 48 h ↓ levels in melatonin treated at 48 h
Jing <i>et al</i> <sup>[63]</sup>	C57B/16 male mice	Contusion	IH Impactor	50 kdyn	T10	Melatonin, 10 mg/kg	Twice/day, 2 wk post injury		↑ AQP4 in untreated at 7 d ↓ AQP4 in melatonin treated at 7 d	↑ AQP4 in untreated at 7 d ↓ levels in melatonin treated at 7 d		↑ in melatonin treated at 7, 10 and 14 d (BMS)
Zu <i>et al</i> <sup>[69]</sup>	Sprague dawley male rats	Contusion	Striking bar	150 gcf, 5 cm drop	T8	Curcumin, 40 mg/kg	Once, 30 min post injury	↑ AQP4 in untreated at 72 h ↓ AQP4 in curcumin treated at 72 h	↑ AQP4 in untreated at 72 h ↓ AQP4 in curcumin treated at 72 h	↑ AQP4 in untreated at 72 h ↓ AQP4 in curcumin treated at 72 h		↑ levels in untreated at 72 h ↓ levels in curcumin treated at 72 h
Hu <i>et al</i> <sup>[24]</sup>	Sprague dawley female rats	Contusion	NYU weight drop device	10 g weight, 25 mm drop	T10	Myelotomy, 3.5 mm long, 1-1.5 mm deep	Once, 24 h after injury	↑ AQP4 in untreated at 2, 4 and 6 d ↓ AQP4 in myelotomy treated at 4 and 6 d	↑ AQP4 in untreated at 2, 4 and 6 d ↓ AQP4 in myelotomy treated at 4 and 6 d	↑ AQP4 in untreated at 2, 4 and 6 d ↓ AQP4 in myelotomy treated at 4 and 6 d		↑ levels in untreated at 4 and 6 d, not at 2 d ↑ in myelotomy treated at 14, 21, 28, 35 and 42 d
Mao <i>et al</i> <sup>[68]</sup>	CD1 male mice	Compression	Vascular clip	10 g force, 1 min hold	T9	SFN, 5 mg/kg	Once, 1 h post injury	↓ AQP4 in untreated at 48 h	↓ AQP4 in untreated at 48 h	↓ AQP4 in untreated at 48 h		↓ levels in myelotomy treated at 4 and 6 d
Fan <i>et al</i> <sup>[65]</sup>	Sprague dawley male rats	Compression	Weight application	5 g weight, 5 min hold	T12	AG, 150 mg/kg	Once, 24 h post injury	↑ AQP4 in SFN treated at 48 h	↑ AQP4 in SFN treated at 48 h ↑ AQP4 in untreated at 24 h and 48 h ↓ AQP4 in AG treated at 24 h and 48 h	↑ AQP4 in SFN treated at 48 h ↑ AQP4 in untreated at 48 h ↓ AQP4 in AG treated at 48 h		↓ levels in SFN treated at 48 h ↑ levels in untreated at 12, 24 and 48 h ↓ levels in AG treated 12, 24 and 48 h

Wang <i>et al</i> <sup>[82]</sup>	Sprague dawley male rats	Compression	Weight application	50 g weight, 5 min hold	T12	2ME2, 2.5 mg/kg	Once, post injury	↑ AQP4 in untreated at 6, 12 and 24 h ↓ AQP4 in 2ME2 treated at 12 h
Ge <i>et al</i> <sup>[84]</sup>	Sprague dawley male rats	Compression	Vascular clip	30 g force, 1 min hold	T12	EGCG, 100 mg/kg	1 × /day, 3 d	↑ AQP4 in untreated at 12, 24, 48 and 72 h ↓ AQP4 in EGCG treated at 24, 48 and 72 h ↓ levels in EGCG treated at 24, 48 and 72 h
Liu <i>et al</i> <sup>[58]</sup>	Sprague dawley male rats	Compression	Aneurysm clip	5 min hold	T12	Melatonin, 100 mg/kg	1 × /day, 3 d	↑ AQP4 in untreated at 12, 24, 48 and 72 h ↓ AQP4 in melatonin treated at 24, 48 and 72 h ↑ levels in untreated at 12, 24, 48 and 72 h ↓ levels in melatonin treated at 24, 48 and 72 h

RhEPO: Erythropoietin; TMX: Tamoxifen; AQP4: Aquaporin-4; BBB: Basso, Beattie, Bresnahan; BMS: Basso mouse locomotor scale; NYU: New York University; SFN: Sulforaphane; AG: Aminoguanidine; 2ME2: 2-Methoxyestradiol; EGCG: Epigallocatechin gallate.

protein levels and cord water content from 12 h to 3 d post injury<sup>[80]</sup>. In addition, melatonin administration following compression resulted in improved functional recovery on the BBB locomotor scale and inclined plane tests<sup>[81]</sup>. In all, this supports the neuroprotective and anti-edema effects of melatonin administration following both contusion and compression models of SCI in rats, as previously described. In addition, administration of 2-methoxyestradiol, an HIF-1 $\alpha$  inhibitor, was shown to decrease protein levels of AQP4 following a rat model of compression injury and decrease BSCB permeability by reducing VEGF levels at 12 h following thoracic compression<sup>[82]</sup>. We will not elaborate further on these mechanisms as they have been previously reviewed above.

In agreement with these studies, intraperitoneal injections of aminoguanidine (AG), a small water-soluble compound<sup>[83]</sup>, and the polyphenol epigallocatechin gallate (ECGC) isolated from green tea<sup>[84]</sup> have been shown to decrease AQP4 protein and histological expression, decrease cord water content and improve functional outcome according to the BBB locomotor scale in a thoracic rat compression model. AG is a selective inhibitor of iNOS<sup>[83]</sup>. It may act through attenuation of iNOS activity, reduction of NO formation and preservation of BSCB integrity that is normally compromised following oxidative stress<sup>[83,85]</sup>. Mechanisms by which ECGC administration increases recovery post SCI include: Reduced myelin loss, decreases in apoptotic signaling, increased release of neurotrophic factors and scavenging free radicals<sup>[86,87]</sup>. Attenuation of iNOS activity and inflammation has also been implicated<sup>[88]</sup>.

In contrast, we found one study that concluded that SCI resulted in an initial decrease in AQP4 expression and an increase in AQP4 expression lead to improved outcome. This was the only study found, across all injury models, to report a decrease in AQP4 expression following SCI. Mao *et al*<sup>[89]</sup> found that at 2 d after thoracic compression, AQP4 RNA and protein were downregulated and this downregulation was recovered by administration of sulforaphane (SFN) resulting in increased AQP4 expression and reduced cord water content. SFN is an isothiocyanate present in broccoli that readily crosses the BSCB, and is reported to act as an inducer of antioxidant-responsive element (ARE) genes<sup>[89]</sup>. This is accomplished by activating the nuclear factor-E2-related factor-2 transcription factor, which, upon translocation to the nucleus, binds to ARE in the promoter regions of genes<sup>[90-92]</sup>. In addition, SFN can function to inhibit the nuclear factor- $\kappa$ B pathway to produce an anti-inflammatory effect (reduce pro-inflammatory cytokines) and decrease iNOS activity<sup>[91]</sup>. Coincidentally, the *aqp4* gene promoter region contains AREs<sup>[47,93]</sup>, which accounts for the reason why SFN administration post injury could induce AQP4 upregulation. Lastly, while having no noticeable effect on glial scar formation, SFN administration has been found to promote sprouting/sparing of serotonergic fibers and improve hindlimb function<sup>[91]</sup>. Similar results have been found following controlled cortical impact model of brain injury<sup>[90]</sup>. This research has lead to the proposal that SFN helps to reduce cellular edema by attenuating the loss of AQP4 after injury, enhancing the production of antioxidants and detoxifying enzymes for protection against



**Table 3** Studies utilizing aquaporin-4 null mice

Ref.	Species	Model	Device	Parameters	Level	AQP4 IHC	Water content	Behavioral outcome
Kimura <i>et al</i> <sup>[98]</sup>	CD1 AQP4 -/- female mice, AQP4 +/+	Contusion	IH Impactor	60 kdyn	T10		↑ levels in -/- compared to +/+	↓ in -/- compared to +/+ (BMS, foot print analysis, bladder function)
Saadoun <i>et al</i> <sup>[95]</sup>	CD1 and BL/6 AQP4 -/-, AQP4 +/+	Compression	Dumont 5 forceps	1 mm separation, 2 min hold	T6	↑ AQP4 in +/+ SCI at 2 d	↓ levels in -/- compared to +/+	↑ in -/- compared to +/+ (BMS, foot print analysis)
Wu <i>et al</i> <sup>[68]</sup>	CD1 AQP4 -/-, AQP4 +/+	Transection	Louisville Impactor System Apparatus	0.8 mm depth, right hemisection	C4	↓ AQP4 in +/+ SCI at 2 d and 7 d ↑ AQP4 in +/+ SCI at 42 d	↑ levels in both -/- and +/+ at 3 d ↑ levels in -/- compared to +/+ at 3 d	

AQP4: Aquaporin-4; IH: Infinite horizons; BMS: Basso mouse locomotor scale; SCI: Spinal cord injury.

oxidative stress, decreasing pro-inflammatory cytokine accumulation and increasing the integrity of the BSCB<sup>[90,92]</sup>.

Overall, the results following compression SCI were fairly consistent (Tables 1 and 2). Four of the 5 studies reviewed reported increases in AQP4 expression following SCI that were otherwise reduced following treatment. These studies utilized weight application<sup>[82,83]</sup> and clip compression<sup>[80,84]</sup>. All 4 studies were conducted in rat models. The only outlier in this group of studies utilized a clip compression model in mice<sup>[89]</sup>. While each of the studies examined AQP4 expression profiles within 6 and 72 h of injury, the use of mice may be the reason for the observed differences. Along those lines, characterization of AQP4 expression at more chronic time points following injury and treatment in a compression model would add greatly to this body of work.

## AQP4 EXPRESSION FOLLOWING TRANSECTION SCI

From our literature search, only one study was found to utilize a transection model of SCI for analysis of AQP4 expression. In agreement with all contusion studies and the majority of the compression studies previously mentioned, AQP4 was downregulated up to 1 wk, but increased when observed at 6 wk following a unilateral cervical rubrospinal tract (RST) transection<sup>[68]</sup>. It is noteworthy that in addition to being the only transection study, it was also the only study to examine a cervical level injury. Along similar lines, *in vitro* observation of AQP4 expression following scratch injury showed early downregulation at 1, 12 and 24 h<sup>[94]</sup>. In this study, a correlation was also found between activation of the ERK1/2 pathways and AQP4 expression. Application of U0126, a selective blocker of the pathway, blocked the decrease in AQP4 expression after injury<sup>[94]</sup>.

## OUTCOME FOLLOWING SCI IN AQP4 NULL MICE

The unavailability of AQP4 inhibitors and activators

greatly limits current research approaches. The use of AQP4 null mice has become the only way to examine the necessity of AQP4 following SCI (Table 3). Saadoun *et al*<sup>[95]</sup> was the first to published a study characterizing the histological and functional outcomes following compression injury in AQP4 null mice. Results showed that 2 d after thoracic compression injury, mice lacking AQP4 water channels had improved neuronal survival and myelin sparing along with reduced tissue water content and intraparenchymal cord pressure compared to those with AQP4<sup>[95]</sup>. In addition, AQP4 null mice showed improved functional outcome in the Basso Mouse locomotor scale (BMS)<sup>[96]</sup>, inclined plane test and foot print analysis<sup>[95]</sup>. Lastly, mice showed improved sensory outcome shown by spinal somatosensory evoked response measurements<sup>[95]</sup>. These results helped elucidate the role of AQP4 in cytotoxic edema, in that it was responsible for astrocytic swelling<sup>[95,97]</sup>. That being said, it was proposed that the path by which fluid is eliminated from the lesion site is unknown but may involve diffusion along white matter tracks without the need to pass watertight membranes or AQP4 water channels<sup>[23]</sup>. In this case, AQP4 upregulation would lead to cord swelling and therefore greater tissue infarction.

In contrast with these findings, Kimura *et al*<sup>[98]</sup> found that AQP4 null mice show greater levels of demyelination, cyst formation and greater neuronal loss 42 d following a thoracic contusion injury. In human and rat models of SCI, removal of necrotic tissue by phagocytic cells at the lesion epicenter typically leads to the establishment of fluid filled cavities in their place, irrespective of the species size<sup>[99]</sup>. The results obtained by Kimura *et al*<sup>[98]</sup> are interesting, as mouse models of SCI do not typically display cyst formation. Instead, non-neuronal cells (connective tissue matrix) rapidly replace damaged tissue, which retracts by 3 to 8 wk post-injury<sup>[99]</sup>. Under these conditions, any cavitation produced in the mouse spinal cord following SCI is otherwise closed as the ends of the spinal cord are drawn closer to one another<sup>[99]</sup>. In addition, Kimura *et al*<sup>[98]</sup> showed that injured tissue from AQP4 null mice contained increased water content compared to controls at 2 and 4 wk following injury. Lastly, animals lacking

AQP4 showed reduced functional recovery in both the BMS and foot print analysis tasks<sup>[98]</sup>. In opposition to cytotoxic edema produced by compression injury, contusion injury results in vasogenic edema and disturbance of the blood spinal cord barrier, not cytotoxic cell swelling. From this, it appears as though AQP4 expression is required for clearance of vasogenic edema and is necessary for protection following contusion SCI<sup>[97,98]</sup>. Similar trends are seen in AQP4 null mouse models of cerebral edema, although these studies are not discussed in this review.

Finally, Wu *et al.*<sup>[68]</sup> showed that AQP4 null mice with a unilateral RST hemisection had increased spinal cord edema at 72 h compared to wildtype littermates. In addition, there was resulting reduced migration of astrocytes to the lesion (at week 1), greater lesion volume, glial scar formation and cyst volume (at week 6) and increased retrograde axonal degeneration<sup>[68]</sup>. This study is in agreement with the protective role of AQP4 following a model of vasogenic edema and inevitable breakdown of the BSCB.

## SUMMARY AND CONCLUSION

The role and importance of AQP4 following SCI is still not wholly understood or fully explored. In addition, there remains a lack of mechanistic explanation of AQP4 regulation following injury. The unavailability of AQP inhibitors and activators greatly limits current research approaches. The literature collected in this review support the concept that AQP4 expression plays variable and distinct roles in the formation of edema following SCI. Notably, there appears to be a recurring theme to combat hypoxia, reduce free radical production and availability and preserve integrity of the BSCB as soon as possible following traumatic injury, despite the method of mechanical insult. Continued research into interventions aimed at relieving hypoxia and free radical production should effectively alter AQP4 expression, reduce edema formation and ameliorate negative outcomes associated with these processes. In all, the availability of AQP4 water channels during the formation of vasogenic edema (following contusion or transection SCI) appears to play a protective role, while playing a deleterious role during the development of cytotoxic edema (following compression SCI). It should be noted that the combined degree of both cytotoxic and vasogenic edema in either injury model is not well described, but both remain major contributors to the outcome following SCI. Further studies should be pursued to resolve more about the extent of vasogenic and/or cytotoxic edema in different animal models. These results would help to elucidate conflicting findings between studies, both within and across injury models. In the end, understanding the precise role of AQP4 following a wide range of injuries will help to better understand the timing of treatments following human SCI for optimal therapeutic benefit and enhanced neurological outcome.

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