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Reduced brain edema and infarct volume in aquaporin-4 deficient mice after transient focal cerebral ischemia

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Abstract

Aquaporin-4 (AQP4) is a water channel expressed in astrocyte end-feet lining the blood-brain barrier. AQP4 deletion in mice is associated with improved outcomes in global cerebral ischemia produced by transient carotid artery occlusion, and focal cerebral ischemia produced by permanent middle cerebral artery occlusion (MCAO). Here, we investigated the consequences of 1-hour transient MCAO produced by intraluminal suture blockade followed by 23 hours of reperfusion. In nine AQP4^{+/+} and nine AQP4^{-/-} mice, infarct volume was significantly reduced by an average of 39 ± 4 % at 24 hours in AQP4^{-/-} mice, cerebral hemispheric edema was reduced by 23 ± 3 %, and Evans blue extravasation was reduced by 31 ± 2 % (mean \pm SEM). Diffusion-weighted magnetic resonance imaging showed greatest reduction in apparent diffusion coefficient around the occlusion site after reperfusion, with remarkably lesser reduction in AQP4^{-/-} mice. The reduced infarct volume in AQP4^{-/-} mice following transient MCAO supports the potential utility of therapeutic AQP4 inhibition in stroke.

Keywords

AQP4; transient cerebral ischemia; brain edema; blood-brain barrier

INTRODUCTION

Aquaporin-4 (AQP4) is a bidirectional water transporting protein expressed in astrocytes throughout the central nervous system [6, 18]. AQP4 expression is greatest in the plasma membrane of perivascular end-feet in astrocytes lining the blood-brain barrier (BBB). AQP4 provides a major pathway for water entry into the brain across an intact BBB in cytotoxic brain edema, as produced by water intoxication [20]. AQP4 also appears to be involved in

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the removal of excess water from the brain in vasogenic edema, as produced by brain tumors, though the mechanisms involved are not clear because AQP4 functions as a water-selective transporter [19].

An early pathogenic event in ischemic brain injury is cytotoxic brain swelling in which water from the vasculature enters the brain across the BBB and accumulates in astrocytes [16]. Cytotoxic brain swelling in ischemia is the consequence of cellular dysfunction with consequent Na^+/K^+ pump failure, creating an osmotic gradient driving cellular water influx [22]. Downstream effects of cerebral edema and astrocyte swelling include increased extracellular space glutamate and K^+ concentrations, BBB breakdown and inflammation, resulting in neuronal loss [3, 17]. AQP4 has been shown to be the primary water transport pathway across the BBB [25] as well as the astrocyte plasma membrane [23]. As such, its inhibition has been proposed to be of potential therapeutic utility in cytotoxic brain swelling [28].

Here, we tested the hypothesis AQP4 deletion in mice reduces brain swelling and infarct volume in a model of ischemic stroke produced by transient (1-h) middle cerebral artery occlusion (MCAO) followed by 23-h reperfusion. We previously reported greatly improved outcome in AQP4 knockout mice following focal ischemia produced by permanent MCAO [16], and more recently, improved outcome in AQP4 knockout mice following global ischemia produced by transient carotid artery occlusion [1, 10]. Also, several correlative studies reported reduced cerebral edema following ischemia with reduced AQP4 expression, including propofol and edaravone administration, protein kinase C activation [4], hypertonic saline administration [30], and endothelin-1 overexpression [13]. Together, these results provide a robust body of evidence supporting a neuroprotective effect of AQP4 down-regulation or deletion in cerebral ischemia. However, one recent study reported worse outcome in AQP4 deficiency in an ischemia-reperfusion model [31], though the results are difficult to interpret because the mice used manifest marked baseline abnormalities including BBB dysfunction [32], which are not seen in AQP4-deficient mice used in our studies [1, 10, 16, 21] or those generated by the Oslo group [7].

MATERIALS AND METHODS

Transgenic mice

AQP4 knockout (AQP4^{-/-}) mice were generated by targeted gene disruption as described [14]. All experiments were performed on weight-matched littermates (25–30 g) produced by intercrossing of heterozygous mice in a CD1 genetic background. Protocols were approved by the University of California, San Francisco Committee on Animal Research.

Transient focal cerebral ischemia and reperfusion model

Adult male AQP4^{+/+} and AQP4^{-/-} mice were subjected to transient focal cerebral ischemia by intraluminal middle cerebral artery (MCA) blockage with a 5-0 monofilament nylon suture, as described [29]. Mice were anesthetized with 2% isoflurane in 30% oxygen / 70% nitrous oxide using a facemask. Core body temperature was maintained at 37 ± 0.5 °C. Cannulation of the femoral artery allowed for monitoring of mean arterial blood pressure

and arterial blood gases. A 9.5-mm 5-0 surgical monofilament nylon suture, blunted at the end, was introduced into the left internal carotid artery through the external carotid artery stump, and the left common carotid artery was temporarily occluded. After 1-h MCA occlusion blood flow was restored by the withdrawal of the nylon suture and the mice was sacrificed after 23-h reperfusion.

Regional cerebral blood flow (rCBF) was measured in anesthetized mice using a LASERFLO BPM² blood perfusion monitor equipped with a flexible small-caliber probe of 0.7-mm diameter (VASAMEDICS, St. Paul, MN) [29] which gently touch the skull (1 mm anterior, 4 mm lateral to bregma), away from large pial vessels. Laser Doppler Flowmetry values were averaged over 5-s intervals and recorded before ischemia as a baseline, during occlusion, and after reperfusion. Neurological deficit was scored at 30 min after MCA occlusion to verify successful occlusion. A score = 2 indicated successful occlusion [2].

Quantification of infarct volume and cerebral edema

Brains were removed after 1-h occlusion and 23-h reperfusion, and coronal brain slices were cut at 1, 3, 5 and 7 mm away from the frontal pole by using a mouse Brain Matrix (Harvard Apparatus, Holliston, MA). Brain slices were stained with 2% (weight/volume) 2,3,5-triphenyl- tetrazolium chloride (TTC) in 0.1 M PBS (pH 7.4) at 37 °C [24, 29]. Infarct areas and hemispheric contours at each level were outlined by an investigator blinded to the experimental groups using Image J software. Infarct volume and brain edema were determined by integrating the infarct area of different brain slices areas with the use of cylinder and cone rules [24].

Diffusion-weighted magnetic resonance imaging

Imaging was done on a 7-Tesla, 183-mm bore Oxford magnet equipped with SMIS console and Magnex self-shielded gradients, as described [12]. Diffusion-weighted images (DWI) were acquired from a 1.4-mm thick slice approximately at the level of the bregma. After surgical preparation and occlusion, mice were placed supine in an acrylic carriage with the surface coil fixed securely under the head. The head was taped to the carriage to minimize motion artifact. The time from the onset of occlusion and reperfusion to the start of the first image acquisition was less than 2 min. ADC maps were calculated on a pixel-by-pixel basis from each set of four DWIs [15]. A color scale was used to delineate regions of the ipsilateral hemisphere with reduced ADC relative to the contralateral hemisphere.

Evans blue extravasation

A quantitative assay of Evans Blue was used, as described [11]. 2.5 ml/kg of 4% Evans blue (Sigma, St. Louis, MO) in 0.9% saline was injected by tail vein. After one hour circulation, the mice were sacrificed and perfused transcardially with 200 ml of heparinized saline (10 U/ml heparin in 0.9% of saline). The cerebral hemispheres were separated and homogenized in 400 µl of N,N-dimethylformamide (Sigma, MO) and then incubated for 72 h in a water bath at 55 °C. The samples were centrifuged at 1500g for 20 min. The extracted Evans blue dye in the supernatant was quantified by absorbance at 620 nm. Results were expressed as µg of Evans blue per gram of brain hemisphere by comparison with solution standards.

Statistics analysis

Data are presented as the mean \pm SEM, with comparisons between AQP4^{+/+} and AQP4^{-/-} mice done by the Student t-test using Stat-View 4.0 software (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Physiological parameters

Physiological parameters of four AQP4^{+/+} and four AQP4^{-/-} mice undergoing MCAO were shown at Table 1. There were no significant differences among the groups in rectal temperature, MABP, PO₂, PCO₂, and pH, and no differences in each parameter before, during and after MCA occlusion. Regional cerebral blood flow was reduced similarly by 93.5 % and 92.4 % in AQP4^{+/+} and AQP4^{-/-} mice during occlusion, and was restored at 15 min after reperfusion to 44.9 % and 47.1 % of baseline values before occlusion. These results are typical of reported MCAO studies in mice [9, 29].

Reduced infarct volume and water content in AQP4^{-/-} mice

MCA occlusion was carried out in nine AQP4^{+/+} and nine AQP4^{-/-} mice in which the MCA was occluded for 1 h and brains were removed at 24 h (23 h after reperfusion) (Fig. 1A, top). Fig. 1A (bottom) shows TTC staining of brain sections cut at 1, 3, 5 and 7 mm from the frontal pole. There was significant reduction in infarct area in the slice at 3 mm. Total infarct volume determined from multiple slices in each mouse is summarized in Fig. 1B, with data for each mouse shown separately. There was a significant, 44 % reduction in total infarct volume from $39 \pm 4 \text{ mm}^3$ in AQP4^{+/+} mice to $22 \pm 4 \text{ mm}^3$ in AQP4^{-/-} mice. The enlargement of the ipsilateral, ischemic hemisphere (resulting from brain swelling) was less in the AQP4^{-/-} mice, which attributed to 23% less water (Fig. 1C).

Changes in ADC

Representative ADC maps (Fig. 2A) show reduced ADC on the ipsilateral (left) hemisphere corresponding to increased brain water in the intracellular space and an increase in the size of the intracellular compartment at the ischemic core and surrounding penumbra. The lesion area in the ipsilateral hemisphere was defined as the area in which ADC decreased by more than 20% of that in the contralateral hemisphere. As summarized in Fig. 2B, reduced ADC was seen in the first 15 min during occlusion and remained elevated over 60 min of reperfusion in the AQP4^{+/+} mice. Significantly less reduction in ADC was seen in AQP4^{-/-} mice during the reperfusion period. Baseline ADC values were 625 ± 42 vs. $640 \pm 18 \mu\text{m}^2/\text{s}$ in AQP4^{+/+} and AQP4^{-/-} mice, respectively (difference not significant). These values are in agreement with previous reported absolute ADC values in normal mouse brain [26, 27].

Reduced Evans blue extravasation in AQP4^{-/-} mice

Blood-brain barrier permeability in AQP4^{+/+} and AQP4^{-/-} mice was evaluated after 1-h occlusion and 23-h reperfusion by Evans Blue dye extravasation. Fig. 3 shows increased dye extravasation in the ipsilateral hemisphere of mice undergoing MCAO, with significantly reduced extravasation in the AQP4^{-/-} mice. There was little increase in Evans blue dye in

the contralateral hemisphere compared with that in control mice that did not undergo MCAO.

DISCUSSION

The principal finding of this study was significantly reduced infarct volume and brain swelling in AQP4-deficient mice following transient, 1-h focal cerebral ischemia produced by MCA occlusion, following by 23-h reperfusion. These results extend our original demonstration of improved outcome in AQP4-deficient mice following focal ischemia produced by permanent MCA occlusion [16]. In that study there was significantly improved neurological outcome at 24 h, with reduced infarct volume and brain swelling. Our results also extend data obtained in a mouse model of secondary AQP4 insufficiency produced by α -syntrophin knockout, where the knockout mice showed neuroprotection and reduced brain swelling following 90-min MCA occlusion and 23-h reperfusion [2].

Recently, we showed significant neuroprotection in AQP4-deficient mice in models of global cerebral ischemia produced by transient bilateral carotid artery occlusion (BCAO) alone ('2-vessel occlusion') [10], as well as transient BCAO done one day after bilateral vertebral artery cauterization ('4-vessel occlusion') [1]. In the 4-vessel occlusion model cerebral blood flow during BCAO was reduced by > 94%, which models cardiac arrest and resuscitation. In both the 2-vessel and 4-vessel BCAO models there was greatly improved survival and neurological outcome in AQP4-deficient mice, supporting the conclusion that reduced water permeability of the blood-brain barrier and astrocyte plasma membrane in AQP4-deficiency was responsible for reduced infarct volume and improved outcome. Measurements of intracranial pressure following BCAO and studies in brain slices from AQP4-deficient mice exposed to anoxia supported a mechanism in which early cytotoxic brain edema and astrocyte swelling in response to cerebral ischemia triggers a series of events including increased intracranial pressure, reduced extracellular space volume, BBB disruption, oxidative injury and inflammation, leading to cytotoxicity and infarction. We presume that similar neuroprotective mechanisms apply to account for the beneficial effects of AQP4 deletion in the transient focal ischemia studies done here. Another possible contributing mechanism, which will require further investigation, is that the endothelium in AQP4-deficient mice is less susceptible to ischemic injury than that in wild-type mice.

In addition to extending prior work, a motivation in carrying out the study reported here was a conflicting report published recently [31] in which mice were subjected to 5-min, 15-min or 40-min transient MCA occlusion followed by 24 or 72 h reperfusion. That study showed an increased mortality of up to 50% in AQP4-deficient mice during reperfusion. No mice died in the MCAO study here or the study done in alpha-syntrophin knockout mice [2]. The conflicting data of Zeng et al. [31] are difficult to explain; as reviewed previously [20], a number of unusual phenotypes reported in AQP4-deficient mice created by the Nanjing group may be related to marked baseline abnormalities, including BBB dysfunction [32] and sporadic hydrocephalus [5]. No differences were found in our AQP4^{+/+} and AQP4^{-/-} mice in baseline phenotype, cerebrovascular anatomy, blood-brain barrier integrity, and brain histology [16, 21].

DWI was used here as a secondary measure of cerebral edema, as it is sensitive to early changes in brain tissue water distribution during acute ischemia [12, 26]. Hyperintensity on DWI is the result of a reduction in the ADC of water. The mechanisms underlying the ADC decrease following acute cerebral ischemia are thought to be related to an increase in the fraction of intracellular versus extracellular water following anoxic depolarization due to Na-K-ATPase pump failure, producing an increase in the tortuosity of the extracellular space [8]. Reduced ADC reflects intracellular edema and regional lesion severity [27]. The lesser reduction in ADC in AQP4-deficient mice compared to wild-type mice during reperfusion supports an important role of early brain water accumulation of the pathogenesis of cerebral infarction in ischemia.

In conclusion, AQP4 deficiency remarkably reduced infarct volume and brain edema in an established mouse model of transient focal cerebral ischemia produced by transient MCA occlusion followed by reperfusion. These results support prior studies showing improved outcomes in AQP4-deficient mouse models of focal cerebral ischemia produced by permanent MCA occlusion and global cerebral ischemia produced by transient carotid artery occlusion. Together, these results support the potential therapeutic utility of AQP4 inhibition or down-regulation in ischemic stroke.

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Highlights

- Transient focal ischemia and reperfusion injury was produced by one-hour MCA occlusion followed by 23-hour reperfusion
- Infarct volume and brain edema were greatly reduced in AQP4-deficient mice
- The neuroprotective effect of AQP4 deletion suggests the therapeutic utility of AQP4 inhibition in stroke

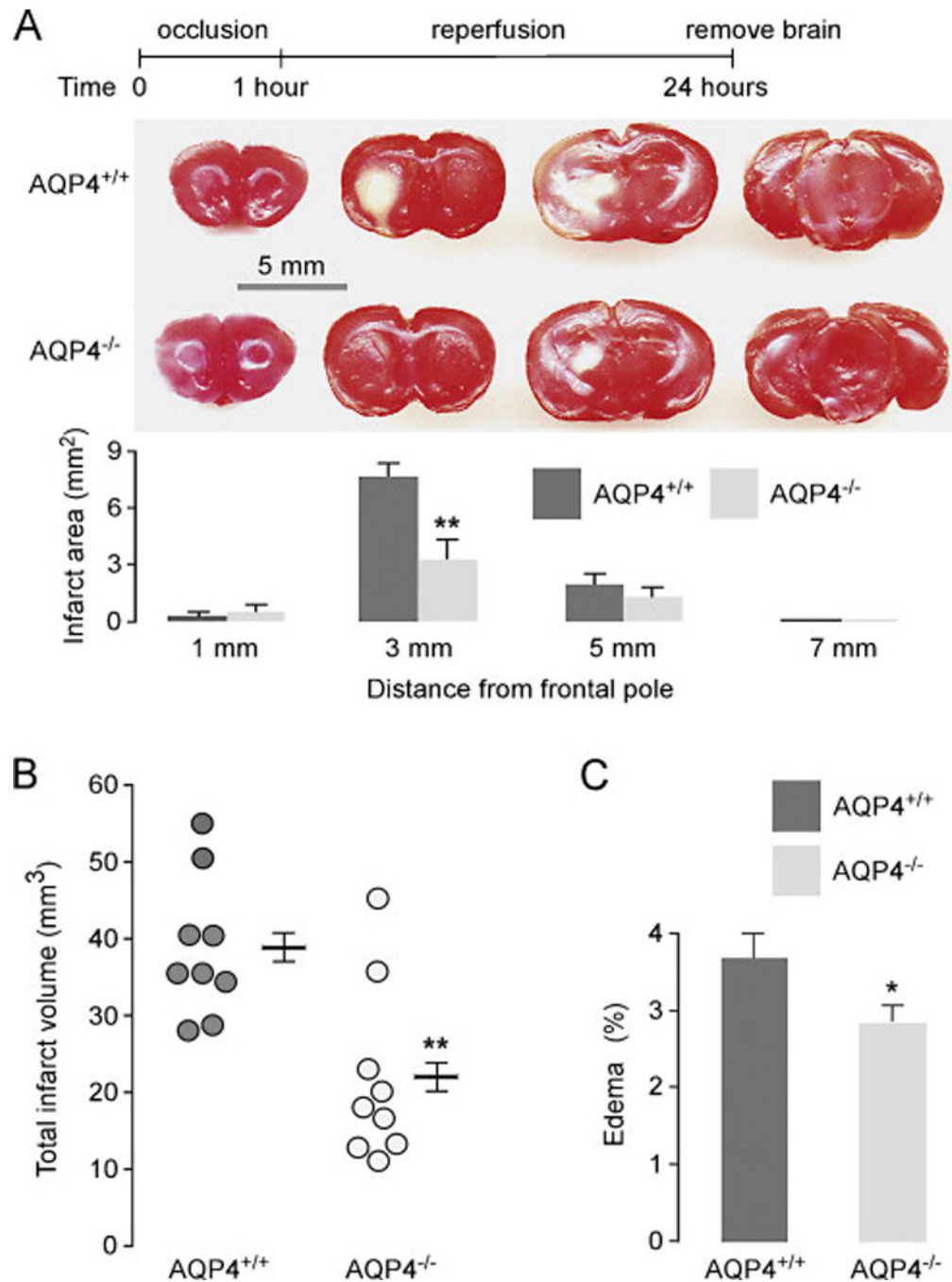


Figure 1. Infarct volume and hemispheric water content at 23-h reperfusion after 1-h MCA occlusion in AQP4^{+/+} and AQP4^{-/-} mice

Brain slices of 2-mm thickness were sectioned at 1, 3, 5, and 7 mm distal from the frontal pole and stained with TTC. A. (top) Diagram of MCA occlusion model. (middle) Representative brain slices showing infarcted area (in white). (bottom) Infarct area (mean \pm SEM, n=9, ** P < 0.01). B. Total infarct volume (from mice in A), **P < 0.01. C. Edema volume deduced from hemispheric enlargement comparing ipsilateral and contralateral brain of mice in A [24]. Edema volume = $(V_i - V_c)/V_c \times 100\%$, where V_i is ipsilateral hemisphere volume and V_c is contralateral hemisphere volume. * P < 0.05.

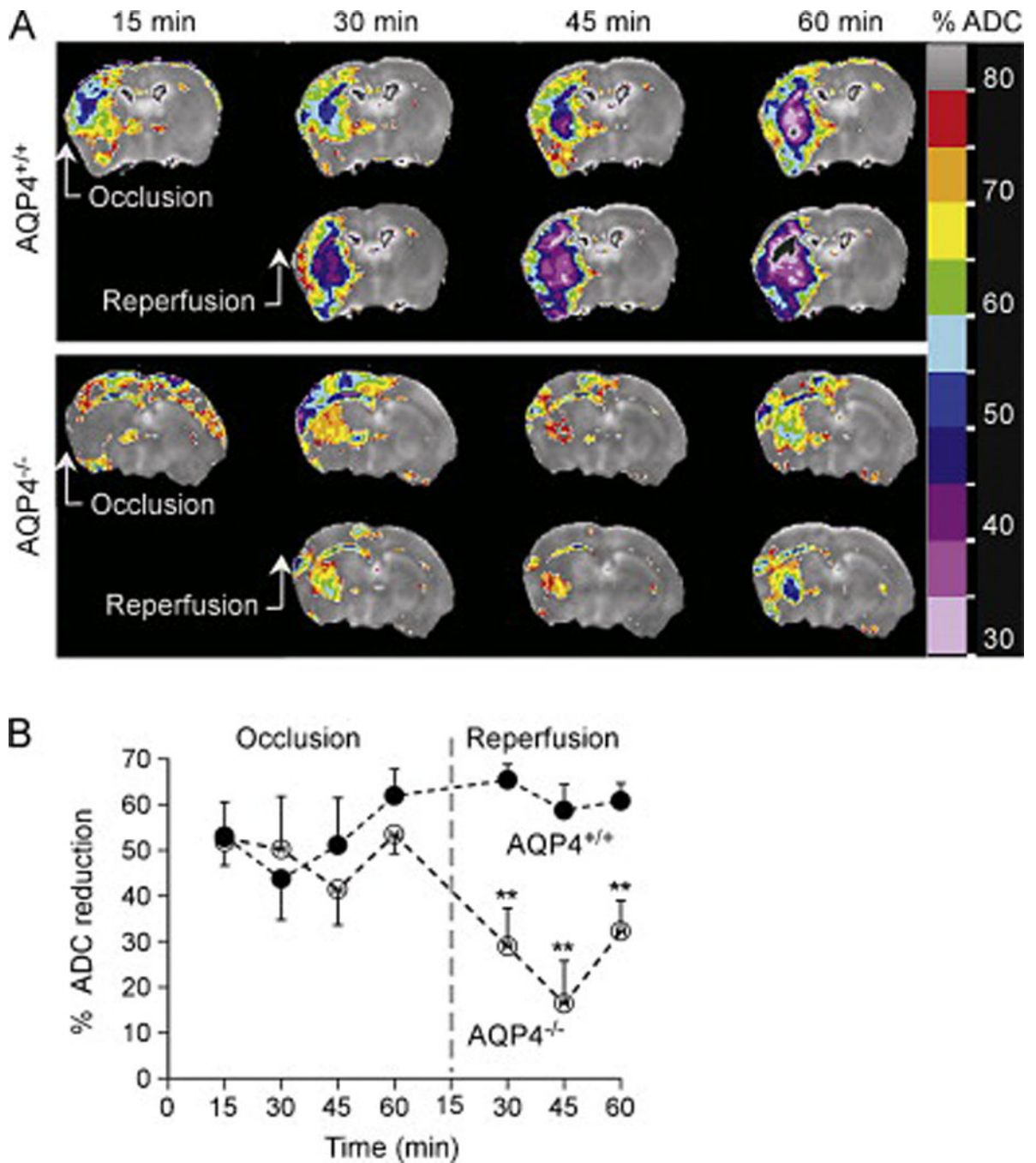


Figure 2. ADC at indicated times during ischemia and reperfusion in AQP4^{+/+} and AQP4^{-/-} mice

A. ADC maps calculated from three adjacent, 2-mm-thick slices, covering the majority of the brain. Representative of maps obtained from four AQP4^{+/+} and four AQP4^{-/-} mice. B. Relative ADC values were calculated by normalizing to the contralateral hemisphere ADC values on a pixel-by-pixel basis, ** P < 0.01.

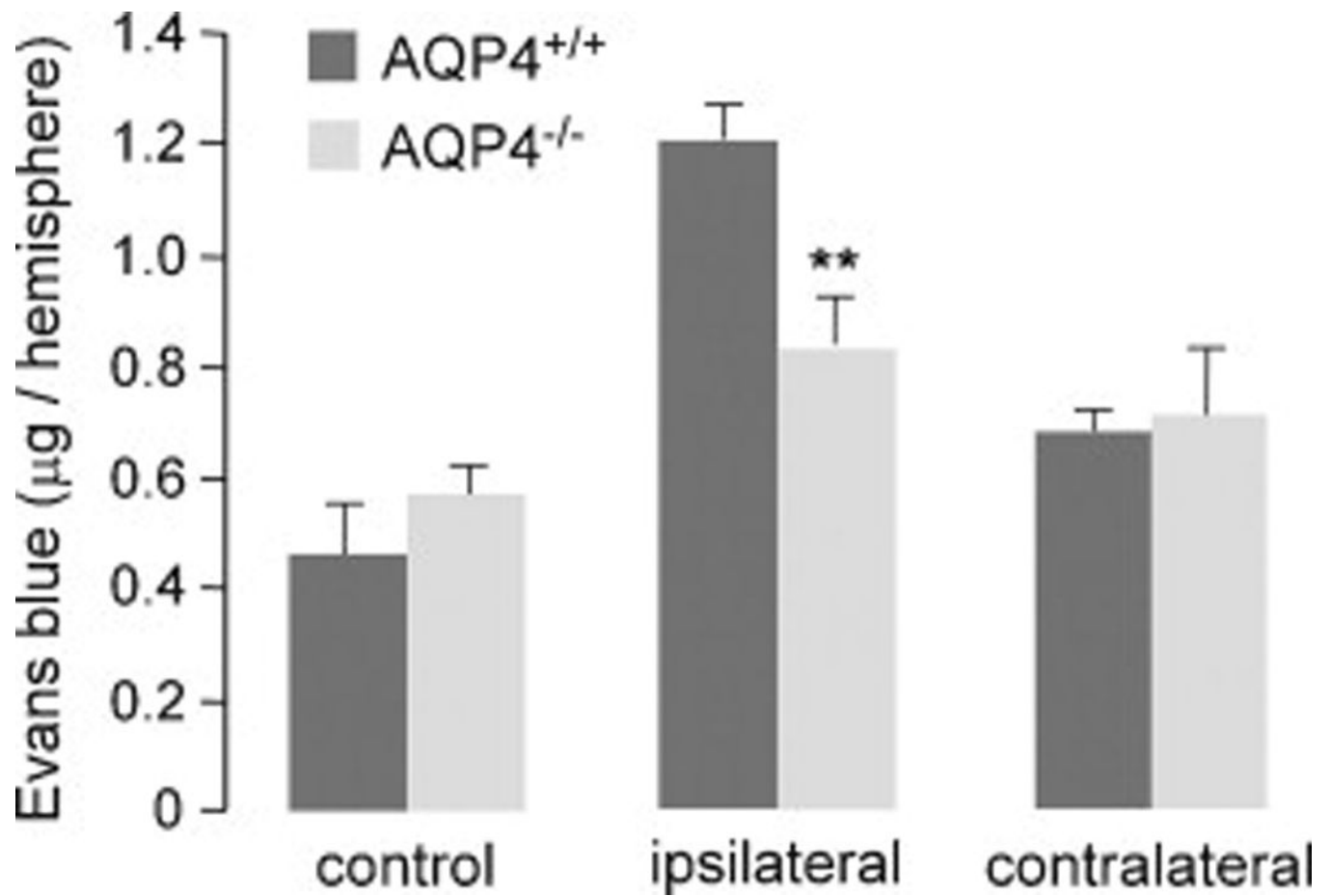


Figure 3. Evans Blue extravasation in AQP4^{+/+} and AQP4^{-/-} mice at 23-h reperfusion after 1-h MCA occlusion

Evans blue dye was injected intravenously 1 h before sacrifice. Dye extravasation was quantified following dye extraction from brain hemispheres after perfusion to remove intravascular dye (mean \pm SEM, 4 mice per group, ** P < 0.01).

Table 1

Physiological parameters of mice undergoing MCAO

	AQP4 ^{+/+}	AQP4 ^{-/-}
P Temperature (°C)	37 ± 0.5	37 ± 0.5
r pH	7.3 ± 0.1	7.4 ± 0.1
e PCO ₂ (mmHg)	31.6 ± 3.2	31.6 ± 3.2
- PO ₂ (mmHg)	124.0 ± 10.3	122.0 ± 9.1
o MABP(mmH ₂ O)	104.0 ± 3.6	97.6 ± 9.2
er CBF (ml _{L,D} /min/100 mg)	104.3 ± 2.7	104.8 ± 4.7
c Baseline ADC (μm ² /s)	625 ± 42	640 ± 18
During occlusion (at 15 min after occlusion)		
u Temperature (°C)	37 ± 0.5	37 ± 0.5
sp H	7.4 ± 0.1	7.4 ± 0.1
i PCO ₂ (mmHg)	35.3 ± 2.5	32.3 ± 3.7
o PO ₂ (mmHg)	97.6 ± 3.0	105.0 ± 10.0
n MABP (mmH ₂ O)	114.3 ± 6.0	122.6 ± 12.2
r CBF (ml _{L,D} /min/100mg)	6.7 ± 0.6	8.0 ± 1.1
After reperfusion (at 15 min)		
Temperature (°C)	37 ± 0.5	37 ± 0.5
pH	7.4 ± 0.0	7.4 ± 0.0
PCO ₂ (mmHg)	35.0 ± 4.5	32.6 ± 5.0
PO ₂ (mmHg)	117.6 ± 13.5	108.3 ± 10.1
MABP (mmH ₂ O)	101.6 ± 10.4	102.6 ± 10.8
r CBF (ml _{L,D} /min/100mg)	46.8 ± 1.1	49.5 ± 6.6

Values are mean ± SEM, 4 mice per group. Differences not significant.

MABP, mean arterial blood pressure; rCBF, regional cerebral blood flow.