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Regional Expression of Aquaporin 1, 4, and 9 in the Brain During Pregnancy

Marchien J. Wiegman, BS, Lisa V. Bullinger, BS, Meghan M. Kohlmeyer, BS, Timothy C. Hunter, MS, and Marilyn J. Cipolla, PhD

Pregnancy is a state of physiologic adaptation, with significant changes in cardiovascular, renal, and hemodynamic systems. Aquaporins (AQPs) may play a role in facilitating these changes. While AQP expression has been assessed in several organs during pregnancy, little is known about its expression in the brain during pregnancy. Therefore, this study assesses the regional expression of AQP1, 4, and 9 during pregnancy and the postpartum period using real-time quantitative polymerase chain reaction. The authors show that AQP1, 4, and 9 are expressed in the anterior and posterior cerebrum, cerebellum, and brainstem of nonpregnant, midpregnant, late pregnant, and postpartum rats. The regional distribution pattern of AQP4 and 9 remained similar during gestation, whereas this pattern changed for AQP1. The expression levels of AQP1, 4, and 9 in the brainstem did not change with gestation, whereas changes were found in the anterior cerebrum for AQP4 and in the posterior cerebrum and cerebellum for all AQPs.

KEY WORDS: Aquaporin, pregnancy, brain.

Aquaporins (AQPs) are a family of transmembrane channel-forming proteins that facilitate water movement across plasma membranes.¹ In addition to water transport, some AQPs also have permeability to small solutes including glycerol, urea, and monocarboxylates.^{2,3} To date, 13 AQPs have been identified in mammalian tissues⁴; however, only 3 have been shown to be expressed in brain in vivo and include AQP1, 4, and 9.⁵

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AQP1 is permeable only to water and is expressed in the epithelial cells of the choroid plexus.^{6,7} Given this location and the finding that AQP1-deficient mice have reduced cerebrospinal fluid (CSF) formation,⁸ AQP1 is proposed to play a role in CSF production.

AQP4 is the most abundant AQP in the brain, and its permeability is restricted to water.⁵ Expression of AQP4 is found in several brain regions, including the cortex, hippocampus, magnocellular hypothalamic nuclei, cerebellum, and brainstem.^{5,9} In these regions, expression of AQP4 is restricted to the endfeet of astrocytes, bordering the ventricles, the subarachnoid space, and the blood vessels.¹⁰ While some studies have reported AQP4 expression in the endothelium,^{11,12} other studies did not find AQP4 at this location.^{10,13} Several physiologic roles have been proposed for AQP4. Given its location at the blood-brain and brain-CSF interfaces, it is thought that AQP4 has a role in cerebral water homeostasis. In addition, co-localization in astrocytes with the potassium channel Kir4.1 suggests that AQP4 may be involved in potassium homeostasis.¹⁴ Lastly, AQP4 expression in the magnocellular hypothalamic nuclei suggests a role for AQP4 in central osmoregulation by transferring variations in plasma osmotic pressure from blood to the osmosensitive neurons in these nuclei.^{10,15}

AQP9 facilitates the diffusion of both water and several small solutes such as glycerol, urea, purines, pyrimidines,

and monocarboxylates.² It is expressed in tanycytes, ependymal cells, and astrocytes lining the ventricles, in astrocytes of the glia limitans, and endothelial cells of pial vessels.^{16,17} The expression of AQP9 at these locations suggests its involvement in cerebral water homeostasis. Moreover, AQP9 expression is found in catecholaminergic neurons, suggesting a role for AQP9 in brain energy metabolism by facilitating transport of small metabolites such as glycerol and lactate.¹⁷

We have previously shown that expression of AQP4 protein is increased in the brain from late pregnant rats.¹⁸ Pregnancy is a state of physiologic adaptation during which significant changes in cardiovascular, renal, and hemodynamic systems occur.^{19,20} These changes include a 45% increase in plasma volume and a 30% to 50% increase in cardiac output.¹⁹ How AQP expression changes during pregnancy to facilitate these adaptations has been studied in several organs. For example, AQP2 mRNA and protein expression are shown to increase more than 100% in rat kidneys during pregnancy.²¹ This upregulation of AQP2 may contribute to the water retention seen in pregnancy. Furthermore, the expression profile of AQP0 through 9 has been assessed in mice uteri during the peri-implantation period since these AQPs may participate in the preparation of the uterus for implantation by facilitating the formation of uterine edema.²² While the effect of pregnancy on these organ systems has been well studied, how pregnancy affects the brain, including AQP expression, is not well understood. Our previous study investigated AQP4 expression in the brain during pregnancy and found a significant increase in AQP4 protein in the whole brain. However, nothing is known about the regional distribution during pregnancy or how the expression of other AQPs in the brain changes during gestation. Therefore, this study investigates the expression of AQPs 1, 4, and 9 in different brain regions during pregnancy and the postpartum state using real-time quantitative polymerase chain reaction (RQ-PCR).

MATERIALS AND METHODS

Animals

For all experiments, female Sprague-Dawley rats (Charles River, St Constant, QC, Canada) were used. All animals were housed in the University of Vermont Animal Care Facility, a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Animals had

access to food and water ad libitum and were maintained at a 12-hour light/dark cycle. All of the procedures were approved by the University of Vermont Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Four different groups of animals were studied: virgin nonpregnant (NP; proestrus stage), midpregnant (MP; day 10 of a 22-day gestation), late pregnant (LP; day 20), and postpartum (PP; day 4).

Real-Time Quantitative Reverse Transcription PCR

To obtain tissue for real-time quantitative reverse transcription PCR (RQ-PCR) analysis, a sample size of 4 to 6 rats was used for each group. Animals were anesthetized with isoflurane (Abbott, North Chicago, IL) and decapitated. Brains were quickly removed from the skull and split into right and left hemispheres. The cerebral cortex, cerebellum, and brainstem were separated from each other; the cerebral cortex was further divided into the anterior and posterior cerebrum by a coronal cut at the level of the optic chiasm. Sections were snap frozen in liquid nitrogen and stored at -80°C until analysis.

For total RNA extraction, brain sections were placed in a FastPrep instrument (MP Biomedicals, Solon, OH) for 45 seconds after adding 1 ml TRIzol Reagent (Invitrogen, Carlsbad, CA) and 0.2 g silicon carbide and aluminum oxide mix (Washington Mills, Niagara Falls, NY). Following centrifugation at 12 000 rcf for 10 minutes at room temperature, cleared homogenate solutions were incubated for 5 minutes at room temperature. After addition of 0.2 mL of chloroform, samples were shaken for 15 seconds, incubated for 2 to 3 minutes at room temperature, and centrifuged for 15 minutes under the same conditions. RNA was precipitated from the aqueous phase by adding 0.5 mL isopropyl alcohol, incubating for 10 minutes at room temperature, and then centrifuging for 30 minutes at 4°C . After washing with 1 mL 75% ethanol, RNA pellets were briefly dried and finally dissolved in RNase-free water. Isolated RNA was then quantified using the NanoDrop spectrophotometer and checked for integrity in a Bioanalyzer (Bio-Rad, Hercules, CA).

Synthesis of cDNA was accomplished by adding 2 μg of total RNA, 1 μL of 50 μM random primers (ABI Research, Oyster Bay, NY), and 1 μL 10 μM dNTP Mix (Invitrogen) in a 13 μL total volume. The mixture was

heated for 5 minutes at 65°C and incubated on ice for at least 1 minute. Next, 4 µL 5X First-Strand Buffer (Invitrogen), 1 µL 0.1 M dithiothreitol (Invitrogen), 1 µL RNaseOUT Recombinant RNase Inhibitor (Invitrogen), and 1 µL of Superscript III RT (Invitrogen) were added to the reaction. Following incubation for 5 minutes at 25°C, the temperature was increased to 50°C for another 30 minutes. The reaction was inactivated by heating to 70°C for 15 minutes.

The resulting reverse-transcribed cDNA was amplified using Assays-on-Demand (AOD) gene expression products kits for AQP1, 4, and 9 (assay IDs Rn00562834_m1, Rn00563196_m1, and Rn00576331_m1; Applied Biosystems, Foster City, CA). One microliter of cDNA was mixed with 10 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µL AOD, and 8 µL water. The PCR was carried out in a 7900HT Sequence Detection System (Applied Biosystems) for 2 minutes at 50°C and 10 minutes at 95°C followed by 15 seconds at 95°C and 1 minute at 40°C for 40 cycles. MapK6 was used as an endogenous control. All samples were run in technical duplicates. Data were analyzed using Sequence Detection 2.2 software (Applied Biosystems).

Comparison of Groups

Two sets of normalization were done to assess both the regional distribution of AQPs at each gestational stage and also how gestation affected expression in the different brain regions. To assess the regional distribution of AQP expression at different gestational ages, data were normalized to the values found for the anterior cerebrum for each AQP in each gestational group. To assess how gestation affected the expression of AQPs within the brain regions, data were normalized to the NP values for each AQP in each region.

Statistical Analysis

All data are expressed as the mean ± SEM. Differences in AQP4 mRNA expression were determined using a Wilcoxon signed-rank test with comparison to a hypothetical value. Differences between unnormalized data were determined using an analysis of variance with a post hoc Student-Newman-Keuls test for multiple comparisons. Differences were considered significant if $P < .05$.

RESULTS

Regional Distribution of AQPs

To assess the regional distribution of AQPs, expression levels were normalized to the levels found for the anterior cerebrum (Figures 1, 2, and 3).

Regional Distribution of AQP1

AQP1 was expressed in all brain regions in all 4 groups of animals (Figure 1). The NP and PP animals showed a similar distribution pattern in the brain, with higher expression in the posterior cerebrum and cerebellum compared with the anterior cerebrum (Figure 1A and D). However, this was significant only in the PP group (Figure 1D; $P < .05$ vs anterior). The MP animals had lower expression in the cerebellum and brainstem compared with the posterior cerebrum (Figure 1B; $P < .01$). The LP animals also had lower expression in the brainstem compared with the posterior cerebrum (Figure 1C; $P < .01$). In addition, the posterior cerebrum revealed higher expression versus the anterior cerebrum ($P < .05$), and there was more expression in the cerebellum compared with the brainstem ($P < .05$).

Regional Distribution of AQP4

AQP4 was also expressed in all brain regions in all 4 groups of animals (Figure 2). Unlike the distribution of AQP1, the distribution pattern of AQP4 within the brain was similar in all groups. All groups showed the highest expression in the brainstem (Figure 2A, NP: $P < .05$ vs anterior and posterior; Figure 2B, MP: $P < .01$ vs anterior and posterior, $P < .05$ vs cerebellum; Figure 2C, LP: $P < .05$ vs anterior, posterior, and cerebellum; Figure 2D, PP: $P < .01$ vs anterior and cerebellum, $P < .05$ vs posterior), followed by the cerebellum (Figure 2B, MP: $P < .01$ vs anterior; Figure 2C, LP: $P < .05$ vs anterior), posterior cerebrum (Figure 2C and D, LP and PP: $P < .05$ vs anterior), and anterior cerebrum.

Regional Distribution of AQP9

AQP9 was also expressed in all brain regions in all 4 groups of animals (Figure 3). Similar to AQP4, the regional distribution of AQP9 in the brain was similar in all groups, with no significant difference between the anterior and posterior cerebrum and less expression in both the cerebellum (Figure 3A, NP: $P < .05$; Figure 3B, MP: $P < .05$ vs anterior, $P < .01$

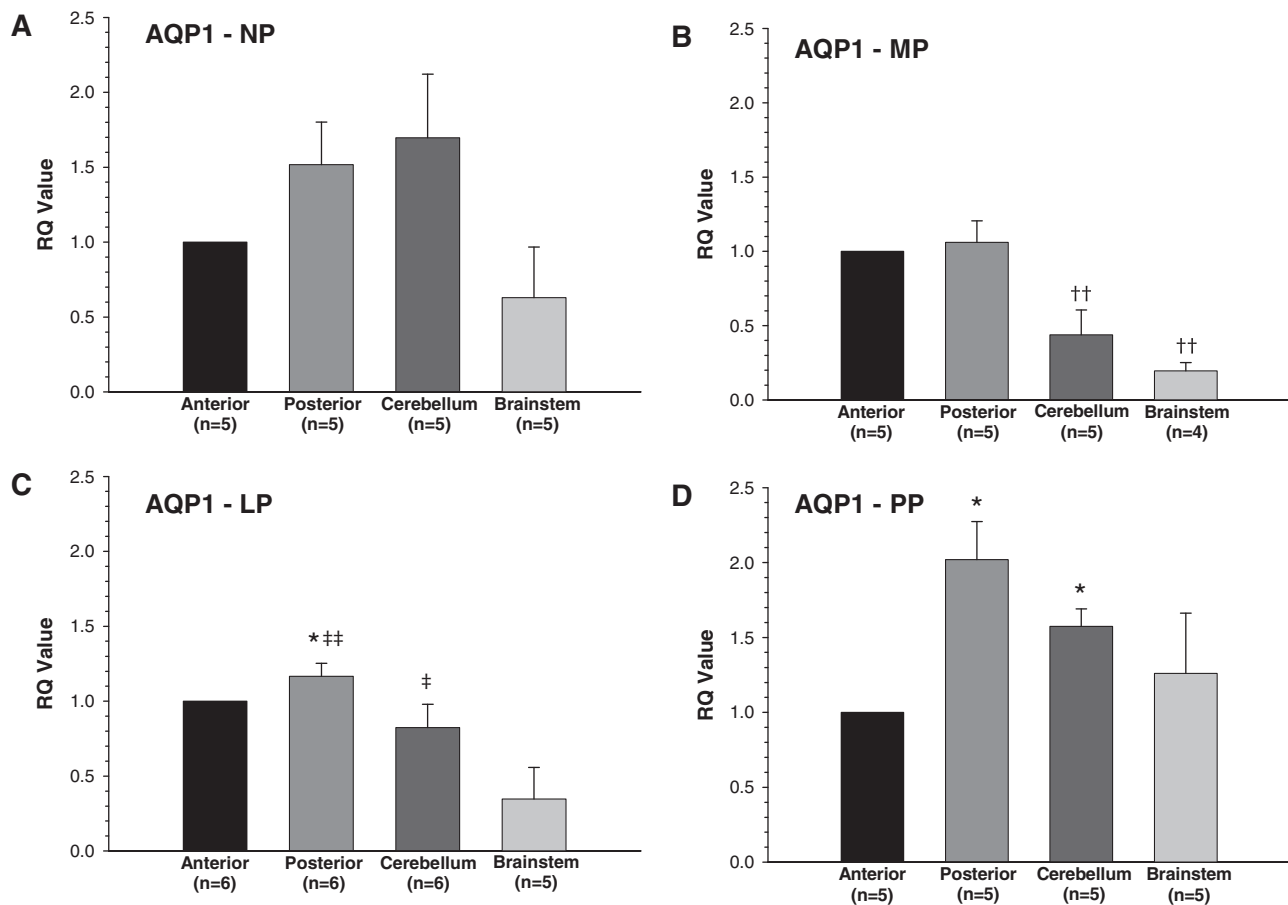


Figure 1. Graphs showing regional aquaporin 1 (AQP1) mRNA distribution between anterior and posterior cerebrum, cerebellum, and brainstem in brains from (A) nonpregnant (NP), (B) midpregnant (MP; day 10), (C) late-pregnant (LP, day 20), and (D) postpartum (PP; day 4) rats. AQP1 expression was normalized to the expression found in the anterior cerebrum. * $P < .05$ versus anterior. †† $P < .01$ vs posterior. ‡ $P < .05$ vs brainstem. ††† $P < .01$ versus brainstem.

vs posterior; Figure 3C, LP: $P < .05$; Figure 3D, PP: $P < .05$ vs anterior, $P < .01$ vs posterior) and brainstem compared with the anterior and posterior cerebrum (Figure 3A, NP: $P < .05$; Figure 3B, MP: $P < .05$ vs anterior, $P < .01$ vs posterior; Figure 3D, PP: $P < .05$ vs posterior).

Gestational Changes in AQP Expression

To assess how gestation affected the expression of AQPs within the brain regions, data were normalized to the NP values for each AQP in each region (Figures 4, 5, and 6).

Gestational Changes in AQP1 Expression

AQP1 expression compared to NP animals did not significantly change with gestation in the anterior cerebrum and brainstem (Figure 4A and D). However, expression did change

significantly in the posterior cerebrum and cerebellum (Figure 4B and C). In the posterior cerebrum, expression decreased in MP and LP compared with NP animals ($P < .05$). Expression in the cerebellum was lower in MP, LP, and PP compared with NP animals ($P < .05$), with the expression in the PP animals being higher than in the MP animals ($P < .01$).

Gestational Changes in AQP4 Expression

AQP4 expression compared to NP animals in the anterior cerebrum did not change in pregnant animals but was lower in PP animals compared with NP animals (Figure 5A; $P < .05$). Expression increased in both the posterior cerebrum and cerebellum in MP and LP animals compared to NP animals (Figure 5B and C; $P < .05$). In the brainstem, no changes in expression were seen with gestation (Figure 5D).

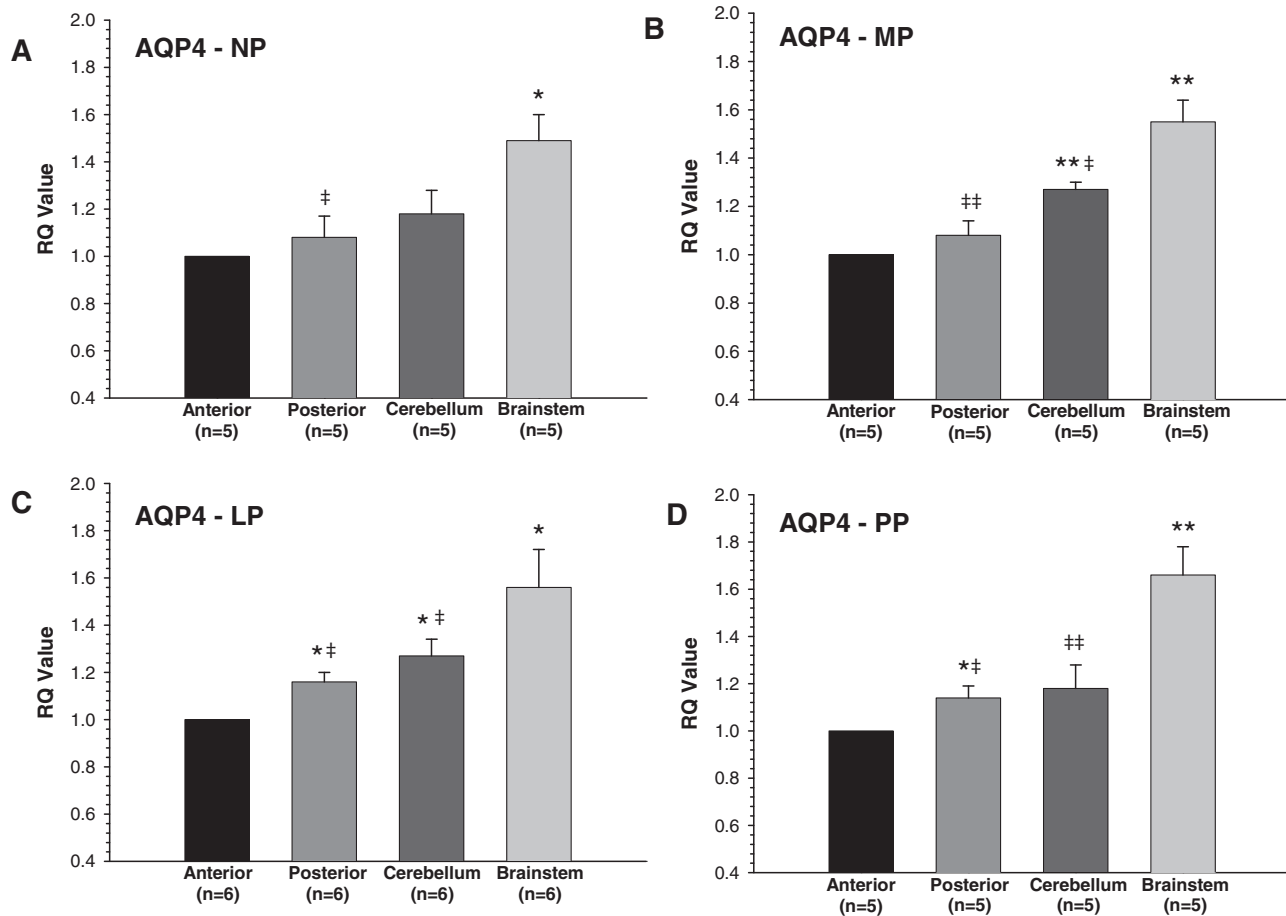


Figure 2. Graphs showing regional aquaporin 4 (AQP4) mRNA distribution between anterior and posterior cerebrum, cerebellum, and brainstem in brains from (A) nonpregnant (NP), (B) midpregnant (MP; day 10), (C) late pregnant (LP; day 20), and (D) postpartum (PP; day 4) rats. Expression of AQP1 was normalized to the expression found in the anterior cerebrum. * $P < .05$ vs anterior. ** $P < .01$ versus anterior. † $P < .05$ versus brainstem. ‡ $P < .01$ versus brainstem.

Gestational Changes in AQP9 Expression

No changes in AQP9 expression with gestation were found in the anterior cerebrum and brainstem (Figure 6A and D). However, expression did change with gestation in the posterior cerebrum and cerebellum (Figure 6B and C). In the posterior cerebrum, expression was decreased in LP versus NP animals ($P < .05$). In the cerebellum, expression decreased in both MP and LP compared with NP animals ($P < .05$) and increased in PP versus MP and LP animals ($P < .01$ vs MP; $P < .05$ vs LP).

DISCUSSION

In this study, we assessed the expression of AQPs 1, 4, and 9 in different brain regions during pregnancy and the postpartum state using real-time quantitative PCR. We

showed that AQP 1, 4, and 9 are expressed in the anterior and posterior cerebrum, cerebellum, and brainstem of NP, MP, LP, and PP rats. The regional distribution pattern of AQP4 and 9 remained similar during gestation, whereas this pattern changed for AQPs 1. The expression levels of AQP1, 4, and 9 in the brainstem did not change with gestation, whereas changes were seen in the anterior cerebrum for AQP4 and in the posterior cerebrum and cerebellum for all AQPs. To our knowledge, this is the first study to assess the regional distribution of AQPs 1, 4, and 9 in the brain during pregnancy.

AQP1 mRNA expression was found in all brain regions, namely, the anterior and posterior cerebrum, cerebellum, and brainstem in all groups (Figure 1), and AQP1 is thought to play a role in the formation of CSF. This is suggested by its location in the epithelial cells of the choroid plexus^{6,7} and the finding that AQP1-deficient

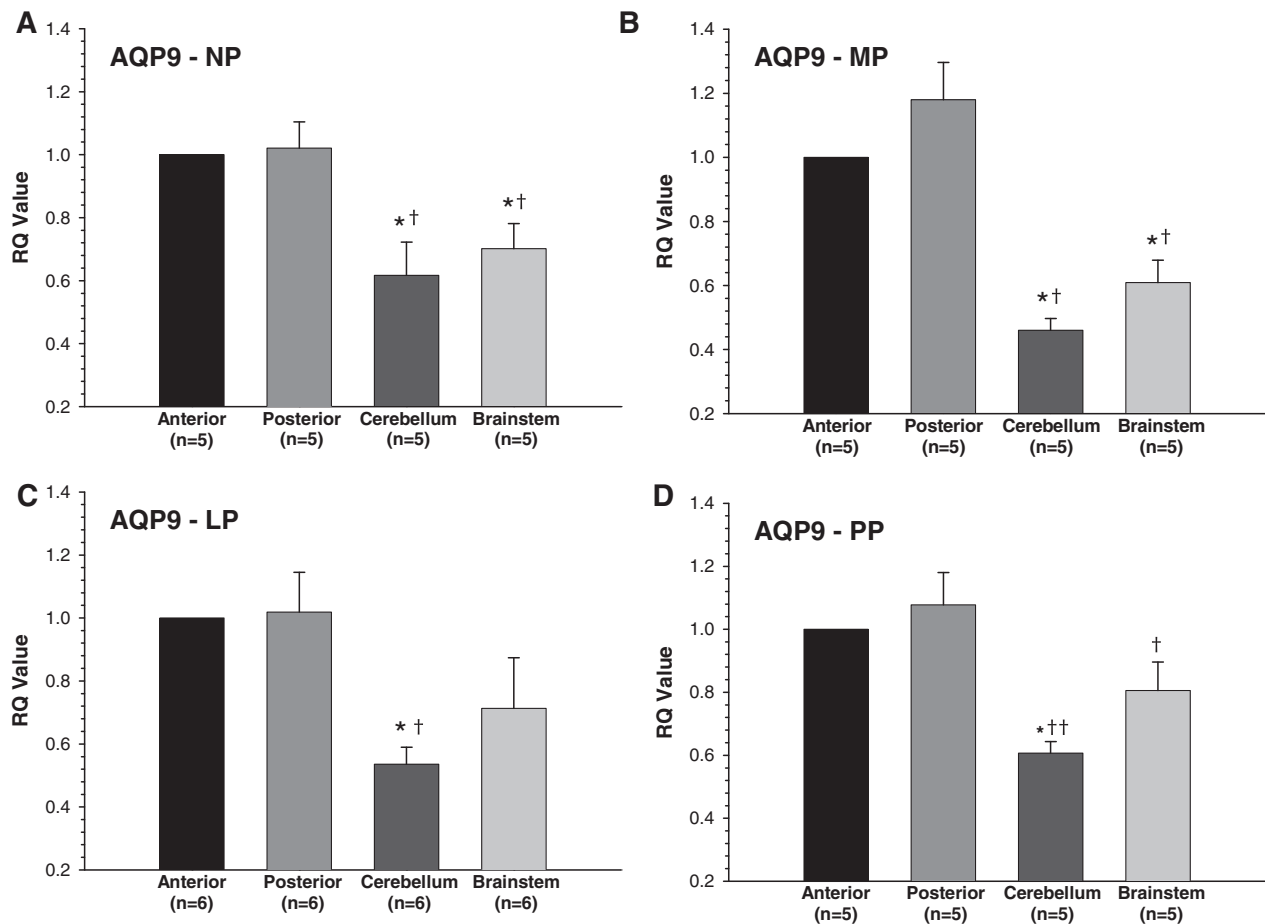


Figure 3. Graphs showing regional aquaporin 9 (AQP9) mRNA distribution between the anterior and posterior cerebrum, cerebellum, and brainstem in brains from (A) nonpregnant (NP), (B) midpregnant (MP; day 10), (C) late pregnant (LP; day 20), and (D) postpartum (PP; day 4) rats. AQP9 expression was normalized to the expression found in the anterior cerebrum. * $P < .05$ versus anterior. † $P < .05$ versus posterior. †† $P < .01$ versus posterior.

mice have reduced CSF formation.⁸ Since the choroid plexus is located in the posterior cerebrum, finding AQP1 in all brain regions suggests that AQP1 expression is not restricted to the choroid plexus epithelium. This is in agreement with results from Dolman et al,²³ who showed the presence of AQP1 mRNA and protein at very low levels in primary rat brain microvessel endothelial cells in culture.

Even though we found AQP1 expression in all brain regions, the relative level of expression is not known. In the NP animals, no difference in expression was found between the regions, although the distribution showed a similar pattern to that of the PP animals, with higher expression in both the posterior cerebrum and cerebellum compared with anterior cerebrum. The MP animals showed a different pattern, with no difference between

the anterior and posterior cerebrum and decreased expression in both the cerebellum and brainstem compared with the posterior cerebrum. In LP animals, a distribution pattern with higher expression in the posterior versus anterior cerebrum and the lowest expression level in the brainstem compared with posterior cerebrum and cerebellum was found. Together, these results suggest that pregnancy changed the distribution pattern of AQP1 regionally in the brain. The significance of this is not clear for this study, and further studies are needed to assess any functional consequence of this gestation-induced redistribution of AQP1.

When assessing gestational changes in AQP1 expression (Figure 4), no significant differences were seen in the anterior cerebrum and brainstem. However, although not significant, the mean expression level in the brainstem

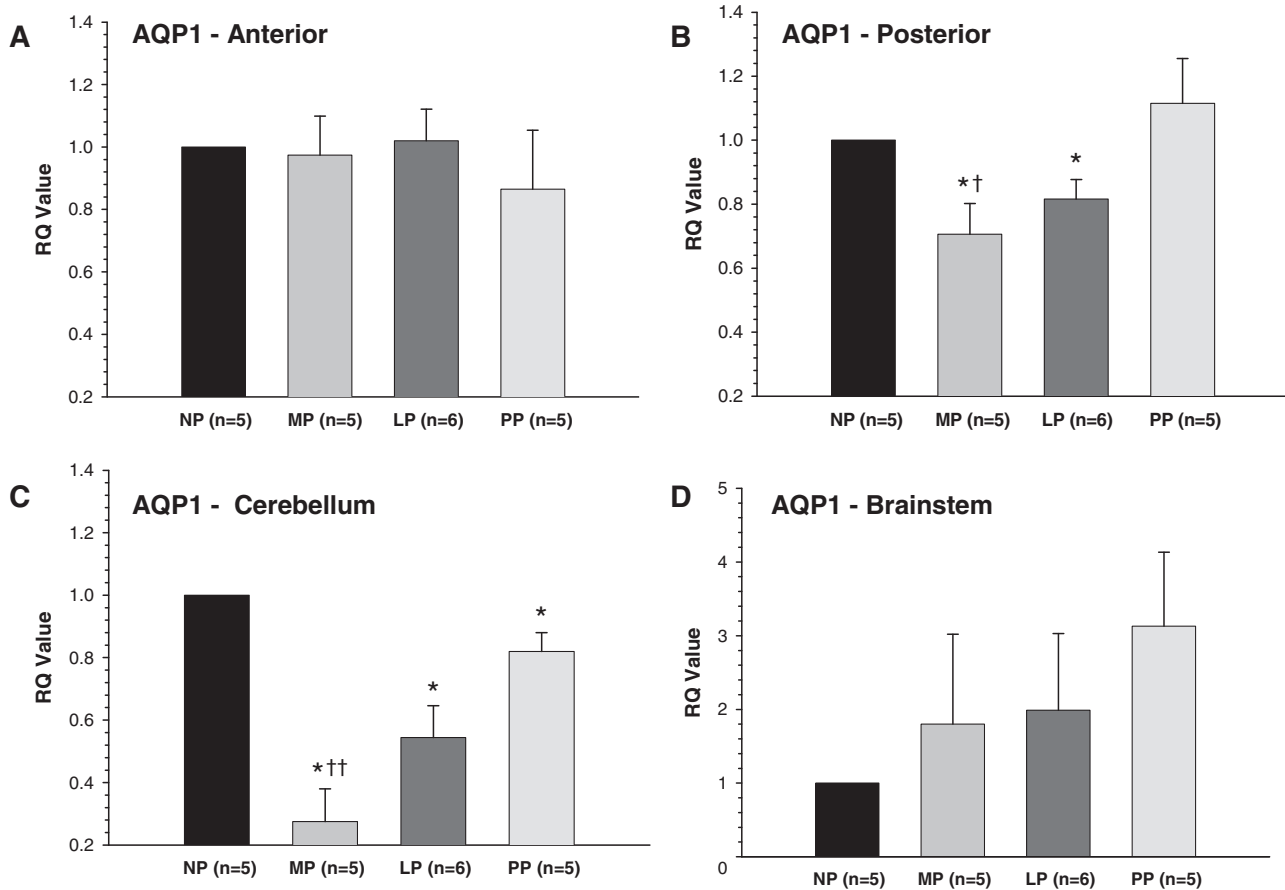


Figure 4. Graphs showing aquaporin 1 (AQP1) mRNA expression levels at different gestational ages in (A) anterior (B) posterior cerebrum, (C) cerebellum, and (D) brainstem. Expression of AQP1 was normalized to the expression found in nonpregnant (NP) animals. * $P < .05$ versus NP. † $P < .05$ versus postpartum (PP). †† $P < .01$ vs PP. LP indicates late pregnant; MP, midpregnant.

increased ~2- to 3-fold during pregnancy and the postpartum period, suggesting that pregnancy does affect the expression of AQP1 in this region. In both the posterior cerebrum and cerebellum, a significant decrease in AQP1 expression was found in brains from MP and LP versus NP animals. Since AQP1 is thought to be involved in CSF formation, the decrease in expression noted in the posterior region during pregnancy may have an influence on this CSF formation. However, it is also possible that expression changes take place at locations other than the choroid plexus since AQP1 expression was found in all brain regions.

AQP4 is the most abundant AQP in the brain and is expressed in several brain regions, including the cortex, hippocampus, magnocellular hypothalamic nuclei, cerebellum, and brainstem.^{5,9} This is in agreement with the current study, which shows AQP4 expression in the anterior and posterior cerebrum, cerebellum, and brainstem

(Figure 2). The same distribution pattern was seen between these brain regions in all gestational groups. AQP4 expression was lowest in the anterior cerebrum, followed by the posterior cerebrum, cerebellum, and brainstem. This expression in the anterior cerebrum and brainstem did not change significantly with gestation, except for a decrease in the anterior cerebrum in PP animals (Figure 5). In the posterior cerebrum and cerebellum, however, expression did change during pregnancy. In both MP and LP animals, increased expression of AQP4 was found in these regions. This is in agreement with our previous study, which found that AQP4 protein expression was increased in LP animals in the whole brain.¹⁸

AQP4 is thought to have several functions in the brain. Given its location at the blood-brain and brain-CSF interfaces, it is thought that AQP4 has a role in brain water homeostasis and in cerebral edema formation and resolution under pathologic conditions.²⁴⁻²⁷ In several

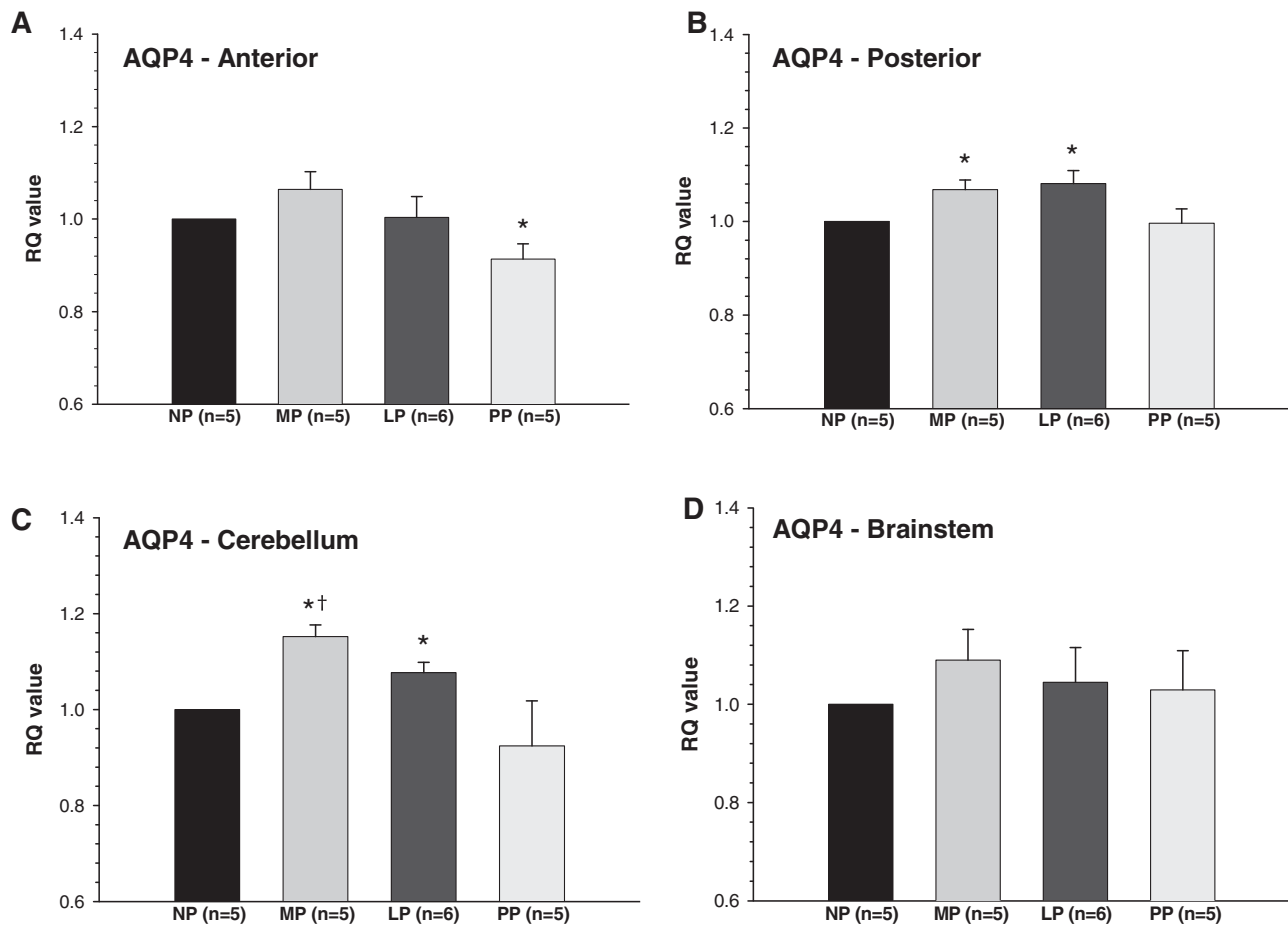


Figure 5. Graphs showing aquaporin 4 (AQP4) mRNA expression levels at different gestational ages in (A) anterior and (B) posterior cerebrum, (C) cerebellum, and (D) brainstem. Expression of AQP4 was normalized to the expression found in nonpregnant (NP) animals. * $P < .05$ versus NP. † $P < .05$ versus postpartum (PP). LP indicates late pregnant; MP, midpregnant.

brain disorders associated with cerebral edema, regulation of the level of AQP4 expression was found, including cerebral ischemia,²⁴⁻²⁷ brain tumors,²⁸ and brain trauma.²⁹⁻³¹ Furthermore, a study using AQP4 knockout mice found that these mice had less edema following acute water intoxication and ischemic stroke compared with wild-type mice, suggesting involvement of AQP4 in edema formation.³² On the other hand, involvement of AQP4 in the resolution of vasogenic edema has also been suggested.³³ Thus, although the role of AQP4 in cerebral edema formation is not completely clear, being the most abundant AQP in the brain, its role in edema formation may be important under pathologic conditions.

One pathologic condition during pregnancy that involves cerebral edema formation is posterior reversible encephalopathy syndrome and eclampsia. Eclampsia is a serious complication of pregnancy in which neurologic

symptoms arise from the development of vasogenic brain edema following an acute elevation of blood pressure.³⁴ Since edema formation in eclampsia is mainly found in the posterior brain regions,^{35,36} it is interesting that we found higher expression of AQP4 in the posterior versus the anterior cerebrum in both LP and PP animals, 2 states during which eclampsia usually develops.^{34,37}

AQP4 is also expressed in the magnocellular nuclei of the hypothalamus, and a role for this water channel has been proposed in central osmoregulation by transferring variations in plasma osmotic pressure from blood to the osmosensitive neurons in these nuclei.^{10,15} However, we did not find any changes in expression in the brainstem, where the hypothalamus is located, with gestation. AQP9 is the only aquaporin in the brain that facilitates diffusion of both water and several small solutes such as glycerol, urea, purines, pyrimidines, and

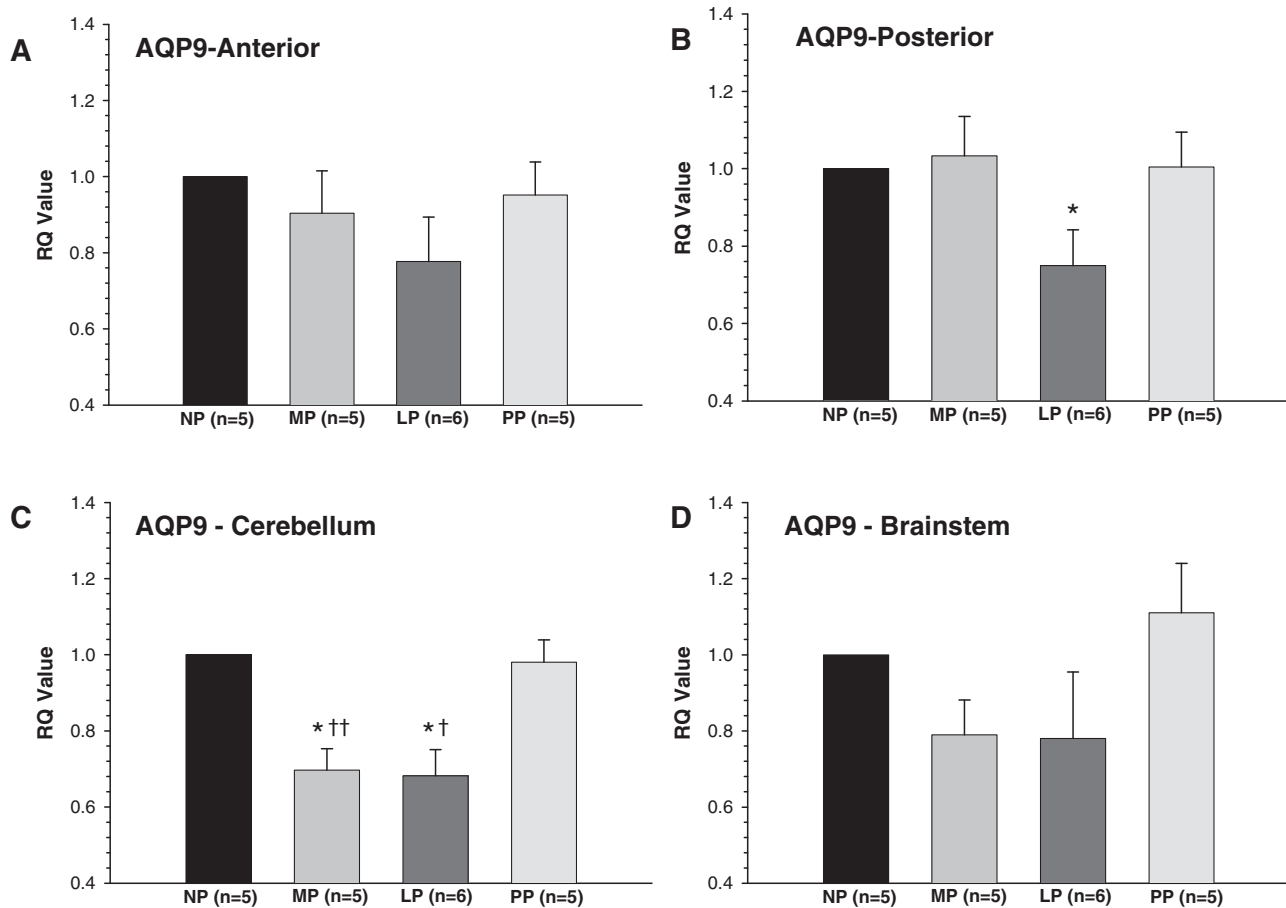


Figure 6. Graphs showing aquaporin 9 (AQP9) mRNA expression levels at different gestational ages in (A) anterior and (B) posterior cerebrum, (C) cerebellum, and (D) brainstem. Expression of AQP9 was normalized to the expression found in nonpregnant (NP) animals. * $P < .05$ versus NP. † $P < .05$ versus postpartum (PP). †† $P < .01$ versus PP. LP indicates late pregnant; MP, midpregnant.

monocarboxylates.² Expression is found in tanyocytes, ependymal cells and astrocytes lining the ventricles, astrocytes of the glia limitans, and endothelial cells of pial vessels.^{16,17} Like AQP4, this localization suggests involvement of AQP9 in cerebral water homeostasis. Moreover, AQP9 expression is found in catecholaminergic neurons, suggesting a role for AQP9 in brain energy metabolism by facilitating transport of small metabolites such as glycerol and lactate.¹⁷

We found AQP9 in all brain regions investigated, corresponding to the locations described previously (Figure 3). All gestational groups revealed a similar pattern of distribution between the regions, showing no significant difference between the anterior and posterior cerebrum and a lower expression level in both the cerebellum and brainstem compared with the anterior and posterior cerebrum. When assessing the changes in AQP9 expression with gestation (Figure 6), no significant changes were

found in the anterior cerebrum and brainstem. However, expression in the posterior cerebrum decreased in LP versus NP animals, and in the cerebellum, expression was decreased in MP and LP versus NP. These decreased levels went back to the NP levels in PP animals, suggesting a direct effect of pregnancy on this AQP.

It is worth noting that we assessed the expression of AQPs 1, 4, and 9 using RQ-PCR, which is the most sensitive and accurate method to assess changes in mRNA expression. While other techniques such as Western analysis for protein levels and immunohistochemistry may provide further information regarding changes in AQPs in the brain during gestation, the results from this study provide a basis of our understanding of how pregnancy alters AQP gene expression in the brain. Because of the large number of groups and brain regions, we did not pursue other techniques but focused on expression-level changes for which other studies can follow.

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