Lithium Causes G2 Arrest of Renal Principal Cells

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ABSTRACT
Vasopressin-regulated expression and insertion of aquaporin-2 channels in the luminal membrane of renal principal cells is essential for urine concentration. Lithium affects urine concentrating ability, and approximately 20% of patients treated with lithium develop nephrogenic diabetes insipidus (NDI), a disorder characterized by polyuria and polydipsia. Lithium-induced NDI is caused by aquaporin-2 downregulation and a reduced ratio of principal/intercalated cells, yet lithium induces principal cell proliferation. Here, we studied how lithium-induced principal cell proliferation can lead to a reduced ratio of principal/intercalated cells using two-dimensional and three-dimensional polarized cultures of mouse renal collecting duct cells and mice treated with clinically relevant lithium concentrations. DNA image cytometry and immunoblotting revealed that lithium initiated proliferation of mouse renal collecting duct cells but also increased the G2/S ratio, indicating G2/M phase arrest. In mice, treatment with lithium for 4, 7, 10, or 13 days led to features of NDI and an increase in the number of principal cells expressing PCNA in the papilla. Remarkably, 30%–40% of the PCNA-positive principal cells also expressed pHistone-H3, a late G2/M phase marker detected in approximately 20% of cells during undisturbed proliferation. Our data reveal that lithium treatment initiates proliferation of renal principal cells but that a significant percentage of these cells are arrested in the late G2 phase, which explains the reduced principal/intercalated cell ratio and may identify the molecular pathway underlying the development of lithium-induced renal fibrosis.


Lithium is widely used as a treatment for bipolar disorder, a common chronic psychiatric illness typically requiring treatment for the rest of the patient’s life. An important side effect of lithium treatment, however, is nephrogenic diabetes insipidus (NDI), a disorder in which urine concentration is impaired, resulting in polyuria and polydipsia.1 Although lithium treatment for a period of weeks already reduces urine concentrating ability in humans,2 approximately 20% of patients receiving long-term lithium therapy will develop clinically extreme concentration defects resulting in NDI.3 Nevertheless, cessation of lithium therapy is usually not an option because bipolar disorder has a larger effect on the patient’s quality of life than NDI. Moreover, due to its efficacy, toxicity profile, and low cost, lithium remains the preferred therapy for bipolar disorders.4

Urine concentration is regulated by arginine vasopressin (AVP), which is released from the pituitary in response to hypovolemia or hypernatremia. In the kidney, AVP binds its type-2 receptor at the basolateral membrane of principal cells of the collecting duct, leading to the redistribution of aquaporin (AQP)-2 water channels from intracellular vesicles to the apical membrane. Driven by the transcellular osmotic

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gradient, water then enters the cell via AQP2 and exits through AQP3 and AQP4 in the basolateral membrane, resulting in correction of the water deficit and in concentrated urine.\textsuperscript{5}

On the basis of studies in rodents, the development of lithium-induced NDI is thought to occur in two phases. In the first short-term phase, lithium causes a decrease in AQP2 expression.\textsuperscript{6} Lithium mainly enters principal cells through the epithelial sodium channel at the apical surface\textsuperscript{6,7} and, consequently, accumulates in principal cells due to the low affinity of the basolateral Na\textsuperscript{+} efflux pump Na\textsuperscript{+}/K\textsuperscript{+}-ATPase for lithium.\textsuperscript{6,8} How lithium downregulates AQP2 remains unclear but likely involves glycogen synthase kinase type 3\beta, which is of importance in AVP-regulated antiurea and is inhibited by lithium.\textsuperscript{9–11} Lithium also affects AQP2-mediated water reabsorption by the elevated tubular release of prostaglandin E\textsubscript{2}.\textsuperscript{11–13}

In a second phase, lithium reduces the percentage of principal cells in the collecting duct, which are “exchanged” for intercalated cells, involved in acid-base homeostasis.\textsuperscript{14} Paradoxically, but in line with increased Wnt/β-catenin induced activity,\textsuperscript{15,16} lithium is known to induce proliferation of principal cells.\textsuperscript{17,18} Apoptosis or principal-to-intercalated cell conversion could be explanations, but Christensen \textit{et al.} concluded that the number of detected apoptotic events or cells costaining for principal and intercalating cell marker proteins in lithium-induced NDI rats was too low to support these explanations.\textsuperscript{17}

In this study, we provide an explanation for this paradox.

\section*{RESULTS}

\subsection*{Lithium Initiates Proliferation of Mouse Renal Collecting Duct Cells}

To study lithium-induced NDI \textit{in vitro}, mouse renal collecting duct (mpkCCD) cells were cultured on two-dimensional (2D) transwell filters as described\textsuperscript{6,19} and treated with 1 and 10 mM lithium at the basolateral and apical side, respectively, for 4, 7, and 11 days. Immunoblotting showed significant downregulation of the 1-deamino-8-D-arginine vasopressin (dDAVP)–induced expression of endogenous AQP2 at all time points (Figure 1A). The effect of lithium on mpkCCD cells was also studied when grown as spheroids, because this condition mimics the physiologic three-dimensional (3D) structure of the collecting duct.\textsuperscript{20} Immunoblotting and immunocytochemistry revealed that treatment of spheroid-grown mpkCCD cells with 10 mM lithium for 3 days significantly reduced endogenous AQP2 abundance (Figure 1, B and C).

To assess the effect of lithium on proliferation, mpkCCD cell cycle profiles were obtained using DNA image cytomtery (Figure 2A) and immunoblotting. In line with lithium-induced proliferation, the 2D model demonstrated a significant decrease in cells in the G0/1 phase, which was accompanied by a significant increase in cells in the S and G2 phases at all days tested (Figure 2B). Similar data were obtained with 3D-grown cells (Figure 2C). Immunoblotting revealed that in our transwell model, lithium significantly increased the abundance of the S/G2 and G2/M phase marker proteins PCNA and phospho-Histone-H3 (pHistone-H3), respectively, but the effect gradually decreased over time (Figure 3, A and B). The abundance of cyclin-D1, a protein essential for the transition from the G0/1 phase to the S phase and whose proteolysis is mediated by glycogen synthase kinase type 3\beta activity,\textsuperscript{21} was strongly elevated upon lithium treatment, whereas β-actin levels remained constant. With 3D spheroids, the effects of lithium on PCNA, pHistone-H3, and cyclin-D1 levels were similar to 2D-grown mpkCCD cells, but β-actin abundance was significantly reduced (Figure 3, C and D). The reduction in β-actin levels in lithium-treated spheroids is due to a reduced cell number because the total Histone-H3 abundance and area-equivalents of proteins (not shown) were also reduced.

\subsection*{Lithium Initiates Proliferation of Mouse Renal Collecting Duct Cells}

To ensure that cell division occurs flawlessly, the transition from one phase to the next phase of the cell cycle is controlled at the G1/S and G2/M checkpoints.\textsuperscript{22} Because lithium increased the number of cells present in the S and G2 phases, we investigated whether lithium affects the G2/M transition step. We therefore assessed the percentage of cells in the G2 phase relative to all cells in the S and G2 phases. Interestingly, in both cell models, we observed a significant accumulation of cells in the G2 phase after lithium treatment compared with the control situation (Figure 4, A and D). Importantly, this percentage remained significantly elevated at all time points tested (Figure 4A), demonstrating that the G2 cell cycle arrest was sustained for these cells. Subsequent assessment of several proteins essential for G2/M transition revealed that cyclin-B1 and phospho-cyclin–dependent kinase 1 (CDK1) protein levels were significantly increased in both models (Figure 4, B, C, E, and F). Comparison between the two model systems demonstrated that the abundance of cyclin-B1 and phospho-CDK1 gradually declined over time in the 2D model and that the abundance of CDK1 was only elevated in the spheroid culture system.

Because a G2 cell cycle arrest is often coupled with activation of checkpoint kinase 1 (Chk1),\textsuperscript{23} the involvement of Chk1 was studied by use of the selective Chk1 inhibitor CHIR-124. Treatment of mpkCCD cells with 500 nM CHIR-124 for the last 4 days did not affect the lithium-induced reduction of cells in the G0/G1 phase or the increase of cells present in the S phase. However, the lithium-induced increase in cells present in the G2 phase was abolished in cells treated with CHIR-124, demonstrating an essential role for Chk1 in lithium-induced G2 accumulation (Figure 5).

\subsection*{In Vivo Lithium Treatment Induces a G2 Cell Cycle Arrest of Principal Cells}

Our \textit{in vitro} data revealed, besides proliferation, that lithium induced a G2/M phase cell cycle arrest. To investigate whether lithium also caused a G2 cell cycle arrest \textit{in vivo}, mice were treated with or without 40 mmol lithium/kg food for 4–13
days. As anticipated, lithium treatment caused a time-dependent increase in urine volume (Figure 6A) and a decrease in urine osmolality (Figure 6B). To note, for unexplained reasons, the urine volume decreased at 13 days of lithium treatment compared with 10 days but was still significantly increased compared with controls. Immunoblotting confirmed a parallel reduction in AQP2 abundance (Figure 6C). Subsequently, kidney sections were costained with antibodies against AQP4, H+-ATPase, PCNA, and pHistone-H3 to mark principal cells, intercalated cells, proliferation, and the late G2 phase of the cell cycle, respectively. As previously reported, lithium strongly increased the number of cells positive for PCNA, and was most pronounced at the inner stripe of the outer medulla (ISOM). Figure 7A shows a representative staining of the ISOM of mice treated for 96 hours, cells are treated at the basolateral side with 1 mM lithium chloride and at the apical side with 10 mM lithium chloride. After 4, 7, and 11 days of lithium exposure, cells are collected, lysed, and immunoblotted for AQP2 (upper). Quantification is depicted in the lower panel (n=4 for each condition and time point). (B and C) mpkCCD cells are cultured in matrigel and treated with (Li) or without (CTR) 10 mM lithium chloride. After 3 days, cells are lysed and immunoblotted for AQP2 and signal intensities are quantified, corrected for β-actin (n=4 for each condition). (C) Immunocytochemistry of 3D-grown mpkCCD cells. AQP2 expression is visualized in green, whereas α-tubulin and nuclear 4',6-diamidino-2-phenylindole staining are depicted in red and blue, respectively. *P<0.05, significant difference from control (CTR).

**DISCUSSION**

**Lithium Enhances G1/S Cell Cycle Progression**

In this study, the 3D spheroid cell model was used for the first time to study the effect of lithium on AQP2 regulation. It is stated that cells grown in three dimensions (i.e., spheroids) are more like renal tubules and can thereby reach a higher level of epithelial polarity compared with 2D cell culture. However, in our study, the percentage of 2D cells in the S-G2 phase (2%) was more similar to in vivo compared with spheroids (approximately 12%). Therefore, we see the spheroid-grown cells as an alternative model for 2D-grown cells instead of a better model. Lithium treatment of mpkCCD cells grown as a polarized monolayer or as spheroids increased the number of cells in the S and G2 phases. This was accompanied by an enhanced expression of the proliferation markers PCNA and cyclin-D1, which was also found at the later time points of 7 or 11 days. The sustained effect of lithium on cell cycle progression is in line with the progressive decline of collecting duct function and morphology in rodents treated with lithium. In addition to our mpkCCD model, we observed a stimulatory effect of lithium on the initiation of cell division in mice, as demonstrated by the high number of cells positive for PCNA. At days 4 and 7 of lithium treatment a large number of principal cells were positive for PCNA (25% and 58%, respectively), whereas these percentages were much smaller for intercalated cells (4% and 12%) or for both cell types in control mice, in which a negligible number of PCNA-positive cells was found. This is in agreement with earlier findings.
PCNA-positive cells are similar between both cell types at 10 and 13 days. The observation that lithium first initiates proliferation of principal cells and only later of intercalated cells indicates that there might be two different mechanisms by which lithium induces proliferation of both cell types.

Lithium Induces a Chk1-Dependent G2 Cell Cycle Arrest

We show here for the first time that lithium causes a G2 phase arrest of polarized collecting duct cells at clinically relevant concentrations (1 mM basolateral and 10 mM apical). This cell cycle arrest is likely the cause of the reduced β-actin abundance in the lithium-treated spheroids because it prevents the continuation of cell division, leading to a reduced number of cells. Protein levels of cyclin-B1 and CDK1 in the lithium-treated mpkCCD cells were elevated, suggesting that CDK1-cyclin complex formation, essential for cell cycle progression through the G2 phase, was not affected by lithium. Next, we observed that lithium caused an accumulation of the Tyr15-phosphorylated form of CDK1. CDK1 phosphorylation should indeed take place after complex formation; however, this complex will remain inactive until the phosphate is removed. This final and essential step of the removal of the inhibitory phosphate is performed by cdc25 phosphatase, which in turn is negatively regulated by Chk1.27 The accumulation of pCDK1 upon lithium treatment suggests that removal of the phosphate did not take place, supporting a role for the cdc25-Chk1 signaling pathway in lithium-induced G2 phase arrest. Indeed, treatment with 500 nM CHIR-124, a selective Chk1 inhibitor,28 prevented the G2 phase arrest induced by lithium, indicating the involvement of the Chk1 kinase in the lithium-induced G2 phase arrest. These findings are in line with data from Wang et al., who showed that lithium enhanced the activity of Chk1 kinase in 7721 cells.29 How lithium increases Chk1 activity is unknown.

Besides mpkCCD cells, our data indicate that lithium also induced a G2 cell cycle arrest in mice. Using the pHistone-H3 – positive foci as a marker for late G2 phase, we found that 30%–40% of principal cells were in the late G2 phase during the 7–13 days of lithium treatment, which is markedly higher than found in renal tubular cells (approximately 20%) in nontreated male BALB/c mice aged 8–10 weeks.24 Thus, lithium also induced a G2 phase arrest in vivo. The question regarding whether chronic lithium treatment for months or years would lead to a sustained cell cycle arrest was not answered by our study, because the mice received lithium for a maximum of 13 days. However, we did not see any decrease in the percentage of arrested principal cells during the time course of 7–13 days in mice or in cells treated for 4–11 days with lithium. In this respect, Kling et al. demonstrated an increased presence of nuclear variation (irregular size and shape) of collecting duct cells in rats treated with lithium for 3, 9, and 18 weeks.26 These data are consistent with cells arrested in the G2 phase because an altered nuclear variation...
The consequences of such a prolonged G2 cell cycle arrest are not fully understood, although recent investigations of different AKI models indicate that activation of c-Jun NH2-terminal signaling in G2-arrested cells can lead to renal fibrosis due to the upregulation of profibrotic cytokines, including TGF-β1. Because fibrosis and CKD are also observed after long-term lithium treatment, and lithium has been shown to activate c-Jun NH2-terminal signaling and stimulate TGF-β1 production in the collecting duct, the role of G2-arrested cells in lithium-induced fibrosis should be further investigated.

Various studies demonstrated that lithium increased the number of principal cells incorporated with 3H-thymidine or positive for PCNA and concluded that lithium enhances proliferation of principal cells. Our study reveals that PCNA-positive cells are not necessarily proliferating and that additional proof is required to assess whether cells are actually dividing or whether their cell cycle is arrested. We also noted that the small number of PCNA-positive intercalated cells also exhibited a higher percentage of pHistone-H3 positivity than cells with unaffected cell division. Considering the generally accepted view that lithium cannot enter intercalated cells, we do not have an explanation for cell cycle arrest of the intercalated cells. The altered cell polarization/proliferation status of its neighboring principal cells might also possibly affect the intercalated cells. Another explanation may be that these intercalated cells are derived from principal cells, because principal cells can transit to intercalated cells. Perhaps the pHistone-H3–positive intercalated cells were...
originally principal cells, but transformed into intercalated cells upon lithium entry. In line with this hypothesis, a small subset of ISOM collecting duct cells (2.5%) expressed marker proteins of intercalated and principal cells at day 10 of lithium treatment.

The G2 Arrest of Principal Cells May Contribute to Collecting Duct Remodeling in Lithium-Induced NDI

In addition to the loss of AQP2 expression, lithium treatment induces collecting duct remodeling, in which the ratio of principal-to-intercalated cells decreases.\textsuperscript{14,36} We found a tendency for collecting duct remodeling after 13 days of lithium treatment. The paradoxical finding that lithium induces cell proliferation of principal cells, but nevertheless ends up in a reduced principal/intercalated cell ratio, is not understood. Christensen \textit{et al.} were aware of this paradox and thus investigated different options but only sparsely observed apoptosis of principal cells or conversion into intercalated cells during lithium treatment; therefore, the authors excluded these as potential causes for the reduced percentage of principal cells.\textsuperscript{17} Our study provides an explanation for this paradox as the arrest of a large proportion of PCNA-positive principal cells in the late G2 phase, indicating that they do not further divide.

We, however, believe that the G2-phase arrest of principal cells is not the sole factor leading to collecting duct cell remodeling.
remodeling. Previous studies demonstrated that lithium treatment of rats induced an increased number of cells in the collecting duct.26,34,37 The increased number of cells likely constitutes mostly intercalated cells. The question remains as to why intercalated cells proliferate. Interestingly, because lithium treatment is known to cause a metabolic acidosis,38–40 this might be the trigger for intercalated cells to divide to remove excess acid. Accordingly, an acetazolamide-induced metabolic acidosis did indeed stimulate the proliferation of intercalated cells, resulting in similar collecting duct remodeling as observed during lithium-induced NDI.41 Thus, lithium-induced collecting duct remodeling could be explained by a rather stable population of principal cells, which are partly in a cell cycle arrest, and a proliferating population of intercalated cells.

In conclusion, this study demonstrates that lithium not only induces principal cell division in vitro and in vivo, but also, and for the first time, that lithium induces a cell cycle arrest in the late G2 phase, likely involving inhibited Chk1 activity. The lithium-induced cell cycle arrest is likely to contribute to the reduced percentage of principal cells and collecting duct remodeling in developed lithium-induced NDI.

**Figure 5.** Chk1 kinase blockage prevents lithium-induced cell cycle arrest. mpkCCD cells are grown and treated with or without lithium (Li) on Transwell filters for 4 days as described in the legend for Figure 1. Cells treated with lithium are cultured in the presence or absence of 500 nM of the Chk blocker CHIR-124. Cells are then trypsinized, stained, and analyzed by DNA image cytometry (n=5). *P<0.05, significant difference from control (CTR).

**CONCISE METHODS**

**Animal Experiments**

Mice (C57bl6/j) weighing 15–20 g were obtained from Harlan Laboratories (Horst, The Netherlands) and maintained at the Radboud University Medical Centre animal facility. Mice were divided in five groups of three male mice and three female mice. Mice in group 1 (control) received a normal rodent diet (sniff RM-H V1534; sniff Specialdieten GmbH, Soest, Germany) for 7 days. Mice in groups 2–5 received lithium chloride at a concentration of 40 mmol/kg of chow and were euthanized after 4 days (group 2), 7 days (group 3), 10 days (group 4), or 13 days (group 5) of lithium exposure. All mice had free access to water, food, and a sodium chloride block. For the last 48 hours of the experiment, mice were housed in metabolic cages in order to measure water intake and urine output during the last 24 hours. Mice were anesthetized with isoflurane and euthanized by cervical dislocation, after which the kidneys were removed. Urine samples were analyzed for osmolality using a MicroOsmometer Model 3320 (Advanced Instruments, Inc., Norwood, MA). Complete methods are available in the Supplemental Material.

**Cell Culture and Lithium Treatment Assay**

In the 2D filter model, mpkCCD cells were cultured as previously described.6,19 After 72 hours, the cells were treated with 1 nM dDAVP at the basolateral side to induce AQP2 expression. Simultaneously, cells were incubated with 1 mM lithium chloride at the basolateral side and 10 mM lithium chloride at the apical side. After 4, 7, or 11 days of lithium exposure, the cells were either trypsinized, collected in medium, and then used for the DNA image cytometry or were pelleted and prepared for immunoblotting.

In the 3D spheroid model, mpkCCD cells were prepared at a concentration of 12 × 10^5 cells/ml in medium containing 2 nM dDAVP with or without 20 mM lithium chloride, and mixed 1:1 (vol/vol) with matrigel (Becton Dickinson, Bedford, UK). Then, 0.36 ml aliquots were plated into Costar 48-well plates (Corning, USA). Cells were then grown for 4 days, and the number of cells in the spheroids was counted. The results were then analyzed by DNA image cytometry (n=5).

**Figure 6.** Lithium-treated mice develop NDI. Mice are exposed to food without (CTR) or with 40 mM lithium (Li) chloride/kg food for 4, 7, 10, and 13 days. Mice are placed in metabolic cages for 48 hours and urine output (A) and urine osmolality (B) are determined during the last 24 hours (n=6 per time points for CTR and Li 4–10 days; n=5 for Li 13 days). (C) AQP2 immunoblotting data of whole kidneys of control (CTR) and Li-treated mice. The arrow indicates AQP2. *P<0.05, significant difference from control (CTR).
Corning, NY). The cell/matrigel mix was incubated at 37°C for 2 hours to allow gelling. Afterward, the wells were filled with dDAVP-containing medium with or without lithium. After 3 days, the cells were collected in Recovery medium (Becton Dickinson) and used for DNA image cytometry or pelleted and prepared for immunoblotting.

**DNA Image Cytometry**

DNA image cytometry was performed as previously described. In short, after fixation of cells in Böhm fixative and staining with Schiff reagent, DNA image cytometry was performed with Q-path DNA software to determine DNA ploidy of the nuclei (Leica Imaging Systems Ltd, Cambridge, UK).

**Immunoblotting**

Cells were lysed in Laemmli buffer, incubated at 37°C for 30 minutes, sonicated with a Branson Sonifier (Branson Ultrasonics Corporation, Danbury, CT), and analyzed by SDS-PAGE and immunoblotting as previously described.

**Immunocytochemistry**

Culture medium was removed from matrigel-cultured mpkCCD cells and cells were subsequently washed three times (ice-cold PBS, 0.5 mM CaCl$_2$, and 1.0 mM MgCl$_2$) and fixed for 30 minutes at room temperature with 4% w/v paraformaldehyde in PBS. After washing twice with PBS, cells were treated with permeabilization buffer (0.5% v/v Triton X-100 and in PBS with 0.7% w/v gelatin) for 30 minutes. Cells were then incubated at 4°C overnight with primary antibodies (1:50 Rb5 AQP2 and 1:500 α-tubulin; Invitrogen, Camarillo, CA) in permeabilization buffer and after washing three times with secondary antibodies conjugated to Alexa dyes (1:500; Invitrogen, Paisley, UK) for 2–3 hours at 37°C. Finally, cells were mounted in Vectashield Figure 7. Lithium causes a G2 cell cycle arrest of principal cells in vivo. Kidneys of mice treated or not with lithium chloride as described in the legend for Figure 6 are isolated, fixed, paraffin embedded, sectioned, and subjected to immunohistochemistry. Simultaneous staining is done for AQP4 (principal cell, red), H⁺-ATPase (intercalated cell, green), PCNA (nucleus, purple), and pHistone-H3 (G2 cell cycle phase, green). (A) Representative immunohistochemical staining of ISOM area of a mouse treated for 7 days with lithium. (B) Principal-intercalated cell composition (as a percentage of the total number of cells) of the collecting duct of the ISOM of mice treated for 0–13 days (indicated) with lithium and the percentage of PCNA-positive cells in principal and intercalated cells. For each time point, approximately 500 collecting duct cells per mouse for a total of three mice are counted. (C) Percentage of positive pHistone-H3 cells from the PCNA-positive population at days 4–13. (D) Immunohistochemical staining of a kidney after 10 days of lithium treatment. The arrowhead indicates cells that stain for markers of both intercalated and principal cells. *P<0.05, significant difference from the reference value, obtained from a renal cell population of nontreated mice. CD, collecting duct; IC, intercalated cell; neg, negative; PC, principal cell; pos, positive; ref, reference value.
containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and imaged by confocal microscopy (FV1000, Olympus, Center Valley, PA).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described. In short, kidney sections were incubated with blocking reagent (NEN Life Science Products, Zaventem, Belgium) and incubated overnight (O/N) with 1:1000 rabbit anti–p-Histone-H3. Sections were then incubated for 60 minutes with biotin-labeled secondary anti-rabbit antibody (1:1000) and after that for 30 minutes with 1:100 streptavidin-horseradish peroxidase (TSA Fluorescein System; PerkinElmer, Waltham, MA). After incubation O/N with 1:100 mouse PCNA and 1:400 rabbit H+–ATPase (gift from Dr. S. Nielsen, Aarhus University, Denmark) sections were incubated with secondary antibodies conjugated to Alexa dyes (1:1000) for 1 hour and incubated O/N with 1:100 guinea pig AQP4. The next day, sections were washed, incubated with an Alexa-conjugated secondary antibody against guinea pig for 1 hour and incubated for 8 minutes with 1:50 fluorescein tyramide in amplification diluent (TSA Fluorescein System; PerkinElmer). After washing and incubation with 4′,6-diamidino-2-phenylindole (1:10,000) for 30 minutes, sections were embedded in Fluoromount G (Southern Biotech Associates, Birmingham, AL).

**Statistical Analyses**

The difference between groups was tested by the t test and one-way ANOVA corrected by the Newman–Keuls multiple-comparisons procedure. A P value <0.05 was considered statistically significant.

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**DISCLOSURES**

None.

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