Lithium promotes DNA stability and survival of ischemic retinal neurocytes by upregulating DNA ligase IV

Ying Yang1, Nandan Wu1, Sijia Tian1, Fan Li1, Huan Hu1, Pei Chen1, Xiaoxiao Cai1, Lijun Xu1, Jing Zhang1, Zhao Chen1, Jian Ge1, Keming Yu*1 and Jing Zhuang*1

Retinal ischemia is linked to various disorders such as diabetic retinopathy, glaucoma and other retinopathies. For example, with primary glaucoma, the elevation of intraocular pressure (IOP) often results in ischemia that leads to a lack of blood being supplied to the retina, which decreases the supply of oxygen, imbalances ion transport, triggers oxidative stress and finally causes the death of retinal cells.1,2 Primary neurons have suffered ischemia display genomic fragility and show fragmented DNA, both in vitro and in vivo.3–6 Moreover, improper repair or a failure to repair DNA breaks may lead to a ‘domino effect’, causing gene deletions, translocations and missegregation of large chromosome fragments, which may result in cell death or apoptosis.

DNA double-strand breaks (DSBs) repair includes homologous recombination (HR) and nonhomologous end-joining (NHEJ),7 and NHEJ is the predominant pathway for repair of DNA DSBs in postmitosis cells.8 At present, the reasons why DNA repair fails in ischemic neurocytes remains obscure, but hypothetical support of failed NHEJ may be a therapeutic approach for the treatment of retinal ischemic-induced damage.

Clinically, lithium has been used to treat bipolar disorder for more than 60 years. More recently, much evidence from both in vitro and in vivo experimental studies, as well as clinical analyses, demonstrated the neuroprotective effects of lithium against a variety of insults, including glutamate-induced excitotoxicity, ischemia-induced neuronal damage, radiation-induced brain injury and neurodegenerative conditions.9 Our previous study and the other groups also demonstrated in vitro that lithium protects retinal neurocytes against nutrient deprivation-induced apoptosis.10,11 Lithium has emerged as a neuroprotective agent efficacious in preventing apoptosis-dependent cellular death.

The exact mechanisms underlying the neuroprotective effects of lithium are not well defined, but, currently, are thought to be mediated by more than one pathway: transcriptional regulation, inhibition of apoptosis and changes in neurogenesis.12–14 For example, in cultured neural cells and distinct areas of the brain, lithium increases the activity of two prominent transcription factors: activator protein-1 and phosphorylated cyclic AMP-response element binding protein-1 (P-CREB1).15 Lithium inhibition of GSK-3 also regulates the transcription and expression of factors critically involved in neuroprotective, neurotrophic, anti-inflammatory and neurogenic effects.16–18 Moreover, lithium inhibits cell apoptosis by regulating proapoptotic p53, Bax, caspase, HSP70 and cytochrome c release.19–21

In particular, pioneering studies noted that, during hypoxic–ischemic brain injury, lithium confers long-term protection against DNA damage.20,22 It was revealed that lithium...
treatment protected irradiated hippocampal neurons from apoptosis and improved cognitive performance of irradiated mice.\textsuperscript{23,24} Lithium reduced neural progenitor apoptosis in the hippocampus and ameliorated functional deficits after irradiation to the immature mouse brain.\textsuperscript{13} However, the mechanism of DNA repair that lithium targets when it ameliorates neural degeneration is unknown.

Using cultures of primary rat retinal neurocytes and ischemia–reperfusion (I/R) surgery model, we sought to determine whether pretreatment of lithium could protect retinal neurocytes in both \textit{in vitro} and \textit{in vivo} conditions, and we examined the possible underlying mechanisms, focusing on DNA NHEJ repair.

**Results**

Lithium increases DNA NHEJ repair and viability of retinal neurocytes \textit{after serum deprivation \textit{in vitro}}. To elucidate the DNA repair mechanism through which lithium provides neural protection, DNA DSBs and NHEJ efficiency was assayed \textit{in vitro}. Primary retinal neurocytes were cultured and treated with 1.0 mM lithium chloride, and after 24 h were deprived of serum. γ-H2AX is a well-characterized marker of DNA DSBs \textit{in situ} and an early marker of neuronal endangerment following seizures;\textsuperscript{25,26} thus, the DNA breaks in serum-deprived neurons were detected by double staining with γ-H2AX and Map-2. As shown in Figure 1a, γ-H2AX foci were observed in Map-2-positive cells, and while the number of γ-H2AX foci in retinal neurocyte cells cultured in the presence of serum and treated with lithium or vehicle is no different except on the third day, in serum-deprived neurocytes, lithium exposure caused a marked decrease in γ-H2AX staining compared with the control under a serum-free condition from the first day (\(P<0.05\)) (Figure 1b).

To assess NHEJ activity, as described in a previous study,\textsuperscript{24} primary retinal neurocytes were transfected with a NHEJ substrate to assay overall NHEJ (see Figure 1c for the structure of the NHEJ substrate and the strategy for measuring NHEJ). In this assay, a GFP reporter plasmid is linearized with a restriction enzyme and recircularization via NHEJ repair is detectable by GFP expression. In serum-starved retinal neurocytes, lithium promoted significantly more DNA NHEJ repair (2.64-fold greater) compared with control cells (\(P<0.05\)). In contrast, lithium had no effect without serum-free stimulation (Figure 1d).

Consistent with the above results, for neurocytes grown in the presence of serum, lithium treatment also increased viability on the third day. However, in neurocytes deprived of serum, lithium treatment improved cell survival from the first day compared with the serum-starved controls that were not treated with lithium (\(P<0.05\); Figure 1e). Thus, lithium could promote DNA stability and survival of neurocytes when cultured in serum-free medium.

**Serum-free stimulation triggers upregulation of DNA ligase IV in lithium-medicated retinal neurocytes.** Many proteins are involved in DNA NHEJ repair (e.g., ligase IV, Mre11, Ku80), and thus their expression level was tracked in primary retinal neurocytes cultured in full or serum-free medium. Under either condition, the expression of Mre11 and Ku80 was unchanged (data not shown); however, in serum-deprived retinal neurocytes, lithium notably upregulated the mRNA (by 2.3-fold; \(**P<0.001\); Figure 2a) and protein expression of ligase IV (by threefold; \(**P<0.001\); Figures 2b and c). Therefore, serum-free stimulation has been shown to trigger the expression of ligase IV, which is confirmed by double staining (MAP-2 and ligase IV) (Figure 2e).

To confirm that ligase IV is involved in lithium-mediated neural protection during serum deprivation, the ligase IV expression was silenced via small interfering RNAs.\textsuperscript{27} As shown in Figure 2d, this markedly decreased ligase IV expression. In addition, the NHEJ assay showed that lithium promoted DNA NHEJ repair under serum-deprived conditions (the ratio of GFP-positive cells is represented as a histogram). In serum-starved cells treated with lithium, silencing of ligase IV significantly lessened the number of GFP-positive cells (\(**P<0.001, *P<0.05; \text{Figure } 2f\)). Given the drop in NEHJ repair, it was not surprising that the protective effect of lithium in serum-starved cells was reversed by the siRNA (\(P<0.05, **P<0.001; \text{Figure } 2g\)). In addition, the TUNEL assay also indicated that lithium protects serum-starved cells from apoptosis (Supplementary Figure S6C). These results strongly support the assertion that, under serum-deprived conditions, lithium delivers neuroprotection by upregulating DNA ligase IV.

The serum-deprived upregulation of Nrf-1 and lithium-induced increase of P-CREB1 cooperate to promote transcription of ligase IV in retinal neurocytes. The transcriptional regulation of ligase IV has never been studied in mammalian cells, thus we analyzed a series of luciferase reporter constructs driven by various lengths of the ligase IV proximal promoter region (from −1981 to +164). The region between −121 and −301 contains a putative binding site for activating transcription factor (ATF). (Supplementary Figure S1). The region of the ATF promoter between −301 and −121 was scanned \textit{in silico} for transcription factor binding motifs. Two putative ATF sites were detected, at positions −126 to −133 and −172 to −183. The 5′-GTGACGT-3′ motif, at −126, is a P-CREB1 DNA-binding sequence.\textsuperscript{28} The other, at −172 5′-TGCGCCTGCGCA-3′, is a well-known nuclear respiratory factor 1 (Nrf-1) DNA-binding sequence.\textsuperscript{29} To determine the role of the putative ATF sites in ligase IV promoter activation, these sites were mutated (5′-GT GCCGT-3′ to 5′-GTATT-3′; 5′-TGGACTGGCA-3′ to 5′-TGCAATTGCCG-3′; Figure 3a) and the entire promoter region from −601 to +164 was assayed for lithium inducibility in primary retinal neurocytes cultured in serum-free medium, with 1.0 mM lithium.

Three versions of the promoter were compared with the WT sequence: Mt1, which was mutated at the P-CREB binding site (−126′ to −133); mutant 2 (Mt2), which was mutated at the Nrf-1 binding site (−172′ to −183); and Mt3, which was mutated at both (Figure 3a). The promoter activity was compared relative to that of the backbone PGL3-Basic plasmid, showing that the Mt1 and Mt2 mutations significantly reduced the luciferase activity relative to the WT promoter sequence, and mutating both sites reduced transcription by \(\sim 90\%\).
Although the inducibility of Mt1, Mt2 and Mt3 differed slightly, the difference was not statistically significant (Figure 3b). Therefore, these data show that the sites are necessary, but not synergistic, in regulating ligase IV, and that under serum-deprived conditions they confer lithium inducibility.

We measured the expression level of Nrf-1, CREB1, P-CREB1 and ligase IV in primary retinal neurocytes treated with lithium or vehicle by western blotting. In full medium, CREB1 and P-CREB1 were highly expressed in primary retinal neurocytes, and treatment with lithium did not alter the expression of CREB, P-CREB1, Nrf-1 and ligase IV.
Figure 2  Ischemic stimulation upregulates DNA ligase IV expression in lithium-medicated retinal neurocytes. (a) The level of ligase IV mRNA was significantly upregulated in rat retinal neurocytes cultured in serum deprivation medium after treatment by lithium chloride. **P < 0.001. (b) Western blot analysis indicates that lithium treatment upregulates ligase IV expression in rat retinal neurocytes cultured in serum-free medium but does not affect its expression in full medium. (c) The relative expression of ligase IV in retinal neurocytes was quantified by densitometry. Nutritional starvation notably induced the expression of ligase IV in retinal neurocytes. In lithium-treated retinal neurocytes cultured in serum-free medium, ligase IV expression is significantly elevated. **P < 0.001. (d) siRNA oligos were transfected in retinal neurocytes treated with lithium or vehicle, following serum starvation. siRNA silencing markedly decreased ligase IV expression. (e) Double-stained Map-2 (green) and ligase IV (red) showed that ligase IV was expressed in retinal neurocytes. Scale bars: 20 μm. (f) Silencing of ligase IV significantly abolished the elevation of DNA NHEJ repair in retinal neurocytes following serum starvation (Con, 0.10%; Li, 0.172 ± 0.04%; Li+siRNA, 0.120 ± 0.08%). *P < 0.05, **P < 0.001. All error bars represent SEM.

Figure 3  Two sites (−126, −172) are necessary for ligase IV promoter activation in retinal neural cells. (a) Two mutations were introduced into the ATF sites in the −601/+164 constructs. (b) Primary retinal neurocytes were cultured in serum-free medium, with 1.0 mM lithium, and transfected with a ligase IV promoter reporter containing the WT or Mut ATF sites, shown in (a). The levels of luciferase activity were normalized to Renilla luciferase (WT, 2.47 ± 0.56; M1, 0.286 ± 0.11; M2, 0.208 ± 0.072; M3, 0.106 ± 0.029). n = 3 for each group, **P < 0.001. All error bars represent SEM.
Serum deprivation, however, caused a significant decrease in CREB1 phosphorylation but not CREB1, and remarkably induced the expression of Nrf-1, but ligase IV expression was negligible. Pretreating the cells with lithium, however, notably boosted CREB1 phosphorylation under a serum-free condition. These results are consistent with the previous reports that showed serum deprivation in neural cells induces upregulation of Nrf-1 and downregulation of P-CREB1. Long-term treatment with lithium alone does not significantly increase either P-CREB1 levels or CRE-driven gene transcription, this effect is only seen with glutamate or ischemic insult. In retinal neurocytes treated with lithium, robust CREB1 phosphorylation and Nrf-1 highly upregulated ligase IV compared with the control under a serum-free condition. In addition, compared with control cells, lithium-treated cells expressed less Nrf-1 under a serum-free condition. We posit that the decrease in Nrf-1 was the result of lithium-mediated neuroprotection.

CHIP analysis then was used to further examine whether Nrf-1 and P-CREB1 are directly involved in lithium-mediated transcriptional control. At 24 h after treatment with 1.0 mM lithium, retinal neurocytes were cultured in serum-free medium. At 48 h after treatment, chromatin was sonicated into fragments of ~0.5 kb in length (Figure 4b) and precipitated with rabbit antibodies to Nrf-1 and P-CREB1 (or normal rabbit IgG), and PCR amplified a 360- or 305-bp fragment of ligase IV promoter spanning the ATF sites. The amplified PCR fragments were analyzed on a 2% agarose gel. (d) siRNA silencing markedly decreased CREB1 and Nrf-1 expression in retinal neurocytes. In accordance, the siRNA abolished the lithium-stimulated increase in ligase IV expression. (e) Silencing of CREB1 and Nrf-1 significantly inhibited the increase in promoter activity in lithium-treated cells (Con, 1; Li, 2.2 ± 0.47; Li+siRNA, 1.27 ± 0.03). n = 3 for each group, *P<0.05, **P<0.001.
increase of ligase IV expression (Figure 4d). The inducibility of the wild-type p(−601/+164) ligase IV luciferase reporter constructs showed that silencing CREB1 and Nrf-1 could significantly inhibit the increase in promoter activity observed in the wild-type oligos following lithium treatment (*P<0.05, **P<0.001; Figure 4e). Moreover, the luciferase assay also indicated that the promoter activity was enhanced by PKA activators (forskolin) and was inhibited by PKA inhibitors (H89) (Supplementary Figure S2F). Thus, two sites (−126, −172) are necessary for ligase IV promoter activation in retinal neural cells.

Thus, the results above suggest that the serum-deprived upregulation of Nrf-1 and lithium-induced increase in P-CREB1 cooperate to promote transcription of ligase IV in retinal neurocytes. Transcriptional regulation of ligase IV by P-CREB1 is also confirmed by PKA activators (forskolin) and PKA inhibitors (H89) (Supplementary Figures S2C–E). In addition, P-CREB1 is highly expressed in neurocytes cultured in full medium. Therefore, the serum-deprived upregulation of Nrf-1 might trigger transcription of ligase IV, which is confirmed by exogenous Nrf-1 (Supplementary Figures S2A and B).

Retinal expression pattern of Nrf-1, P-CREB1 and ligase IV in vivo suggests that ischemic stimulation triggers lithium-mediated upregulation of ligase IV. To test whether the expression changes observed in cultured cells faithfully reflects the scenario in the live retina, the expression of Nrf-1, CREB1, P-CREB1 and ligase IV in vitro was tracked in rat I/R surgery model (Figure 5A). Over the course of I/R surgery, the P-CREB1, Nrf-1 and ligase IV expression patterns reflected our in vitro results (Figure 4a). In normal, healthy adult retina, the expression of ligase IV and Nrf-1 was negligible, whereas P-CREB1 was highly expressed (Figure 5B and Supplementary Figure S5). Lithium treatment had no effect without I/R stimulation. Similarly, I/R surgery markedly elevated Nrf-1 expression and decreased CREB1 phosphorylation. Notably, in eyes subjected to I/R surgery, lithium pretreatment induced upregulation of P-CREB1 and ligase IV, and decreased Nrf-1.

The expression patterns were tested by immunohistofluorescence, confirming that after I/R surgery, double staining indicated ligase IV, Nrf-1 and P-CREB1 expression colocalized, and was strongest in the inner layer (INL) and ganglion cell layer (GCL) (Figure 5C). Thus, the results in vivo are consistent with that in vitro.

Lithium enhances DNA NHEJ repair and light responses in vivo. We then tracked γ-H2AX foci in a time course in retina subjected to I/R surgery. Here, γ-H2AX foci were more abundant, and γ-H2AX-positive cells were primarily located in retinal GCL and INL (Figure 6A). Moreover, in retina pretreated with lithium, γ-H2AX foci were far less abundant (*P<0.05; Figure 6B). The presence of γ-H2AX foci in retinal GCL and INL agreed with the expression patterns of P-CREB1, Nrf-1 and ligase IV. In addition, TUNEL assay also indicated that lithium significantly decreased the number of apoptosis cells in retina at different time points after I/R surgery (Supplementary Figures S6A and B). Moreover, the rat retina HE staining also showed that lithium protected the thickness of rat retina from getting thinner after I/R surgery (data not shown).

Moreover, the effect of lithium on DNA NHEJ activity was directly tested in the ischemic rat retina. After I/R surgery, the NHEJ substrate, a linearizing pEGFP-N1 was delivered, as described in Materials and Methods. At 48 h after transfection, eyeballs were fixed, sliced and examined for GFP expression. As expected, the RGC layer showed the most activity (Figure 6c), and, with I/R surgery, lithium significantly promoted DNA NHEJ activity (*P<0.05; Figure 6d). Interestingly, lithium also promoted NHEJ even without I/R surgery, improving efficiency by 1.83-fold (*P<0.05). We speculate that this effect is observed because transfection causes mild damage to cells in RGC layer. In addition, in the I/R surgery group, lithium promoted DNA NHEJ activity by 2.4-fold (**P<0.001). I/R surgery also markedly enhanced NHEJ events even without lithium pretreatment (**P<0.001). These results are partly consistent with previous reports showing ischemic stimulation spontaneously activates DNA repair,3,6 and that lithium promotes DNA repair under the pathological, ischemic state.35,36

Finally, scotopic electroretinogram (ERG) was used to examine retinal function. The amplitudes and implicit times of ERG a- and b-waves were examined at different time points after I/R surgery. In comparison with sham eyes, a- and b-wave amplitudes were markedly decreased after I/R surgery. A representative intensity-response series recorded in dark-adapted, sham-, vehicle- and lithium-treated rats is shown in Figure 7. At 12 h after I/R injury, the a-wave amplitudes of vehicle- and lithium-treated rats were similar; in ischemic state.35,36 at 12 h after I/R injury, the a-wave amplitudes were significantly increased for all time points (*P<0.05, **P<0.01) (Figure 7b). Moreover, after surgery, lithium pretreatment also significantly increased the b-wave, for all time points (*P<0.05, **P<0.01) (Figure 7c). Taken together, these results firmly support the idea that DNA breaks occur in the ischemic retina, and pretreatment with lithium promotes NHEJ repair of these DNA breaks. Lithium pretreatment attenuates damage to the retina after I/R surgery.

Discussion
Our results in retinal neurocytes uncover a previously unidentified property of lithium, showing it promotes DNA stability and survival of ischemic retinal neurocytes by upregulating DNA ligase IV. Moreover, ligase IV is transcriptionally regulated by the ischemic upregulation of Nrf-1 and lithium-induced increase P-CREB1 in retinal neurocytes. Importantly, ischemic stimulation triggers the upregulation of ligase IV. All results have been directly tested in the ischemic rat retina. After I/R surgery, the NEHJ substrate, a linearizing pEGFP-N1 was delivered, as described in Materials and Methods. At 48 h after transfection, eyeballs were fixed, sliced and examined for GFP expression. As expected, the RGC layer showed the most activity (Figure 6c), and, with I/R surgery, lithium significantly promoted DNA NHEJ activity (*P<0.05; Figure 6d). Interestingly, lithium also promoted NHEJ even without I/R surgery, improving efficiency by 1.83-fold (*P<0.05). We speculate that this effect is observed because transfection causes mild damage to cells in RGC layer. In addition, in the I/R surgery group, lithium promoted DNA NHEJ activity by 2.4-fold (**P<0.001). I/R surgery also markedly enhanced NHEJ events even without lithium pretreatment (**P<0.001). These results are partly consistent with previous reports showing ischemic stimulation spontaneously activates DNA repair,3,6 and that lithium promotes DNA repair under the pathological, ischemic state.35,36

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Many previous studies have investigated lithium-mediated neuroprotection and its mechanism. Our data show that γ-H2AX foci was observed in retinal cells both in vitro and in vivo with ischemic stimulation. The DNA rejoining levels both in vitro and in vivo reveal that, after ischemia, lithium-pretreated retinal neurocytes showed ~2-fold more DNA repair
(Figures 1d and 6d). Notably, however, lithium did not improve cell viability as much as it promoted DSB repair (Figure 1e). Thus, ischemic stimulation likely damages the cell in ways other than just by inducing DNA DSBs. Still, promoting DNA DSB repair might be one pathway that lithium supports in giving neuroprotection under ischemic condition.

Figure 5  Ligase IV, P-CREB1, CREB1 and Nrf-1 expression pattern in rat retina after I/R surgery. (A) The image of normal rat eye, rat eye at I/R surgery and the rat eye after I/ R surgery. The normal rat eyes have the characteristic of transparency cornea, normal bloodstream in iris and fundus red light reflection (left); the eye at I/R surgery is of corneal edema, interruption of blood flow in fundus and pale retina (middle); the bloodstream of iris and fundus recovery immediately right after the I/R surgery (right). Scale bars: 1000 μm. (B) Western blot shows lithium only alters the expression levels of Nrf-1, P-CREB1 and ligase IV, in retinal tissue subjected to I/R surgery. (C) Location of expression of P-CREB1, Nrf-1 and ligase IV in retina after following I/R surgery. Double staining indicates that ligase IV, Nrf-1 and P-CREB1 are located in same position of retina, mainly expressed in INL and GCL. At 24 h after I/R surgery, sections of lithium-treated retina were double stained with antibodies recognizing Nrf-1 (green) and ligase IV (red) (c1), P-CREB1(green) and ligase IV (red) (c2), and Nrf-1 (green) and P-CREB1 (red) (c3), respectively. Scale bars: 50 μm
Yang et al. reported that lithium-mediated protection of hippocampal cells involves enhancement of DNA-PK-dependent repair in mice but did not identify a particular protein target for lithium. Our study in retinal neurocytes showed that lithium did not directly affect the expression of DNA-PK (data not shown), but rather influenced the expression of ligase IV, a key protein in the predominant pathway for repair of DNA DSBs in postmitotic cells. More importantly, lithium treatment affected the expression level of ligase IV in retinal neurocytes both in vitro and in vivo, under nutritional starvation or an ischemic condition (Figures 2a–c, 4a and 5b). In addition, our data indicated that human retinoblastoma cells did not respond to lithium-mediated retinal neuroprotection by ligase IV. Ligase IV expression in starved WERI-Rb1 cells also did not change with lithium treatment (Supplementary Figure S3).

Therefore, the effect of lithium in enhancing ligase IV was specific to primary neural cells under nutritional starvation or an ischemic condition.

In mice, ligase IV deficiency causes embryonic lethality. Ligase IV is markedly downregulated in the retina of the P5 rat and almost silenced in the adult retina (Supplementary Figure S4). Previous reports on cerebral development demonstrated the DNA repair activity in the young and adult rat, but the expression level declined with age. Nuclear extracts from aged CNS tissue contained NHEJ activity that could be supplemented by exogenously supplied T4 ligase and DNA polymerase b. In addition, ligase IV associates with DNA-PK in a DNA-independent manner. Therefore, our discovery aligns well with earlier reports.

Our discoveries concerning the transcriptional mechanism of ligase IV support the idea that, in these retinal cells, the bioactivity of lithium in promoting DNA NHEJ repair might be triggered by ischemic insult. Our results show that Nrf-1 and P-CREB1 are essential to transcription of ligase IV. CREB is a broadly expressed leucine-zipper-containing transcription factor affecting neuron survival, axon growth, and synaptic transmission. P-CREB may be a more appropriate predictor of CRE-driven transcriptional activity. In nutritionally starved neurocytes, lithium might significantly attenuate a decrease in CREB1 phosphorylation. The other key factor, Nrf-1, is essential for embryogenesis, and embryos homozygous for Nrf-1 disruption die between embryonic days 3.5 and 6.5. Nrf-1 is likely involved in transcriptional regulation of multiple target genes, including those associated with mitochondrial proteins, DNA DSBs and cell cycle. In addition, the NRF
proteins are important for upregulating antioxidant and xenobiotic-metabolizing enzymes and the regulation of neurite outgrowth during oxidative stress.\textsuperscript{44,45} After 36 h of lithium treatment, primary bovine aortic endothelial cells upregulate the mRNA level of Nrf-1 by twofold.\textsuperscript{46} More importantly, our data suggest not only that Nrf-1 and P-CREB1 positively and necessarily promote ligase IV transcription but also that Nrf-1 triggers the process. Our results showed that, in the retina, from first day after birth to adulthood, Nrf-1 and ligase IV display the same expression pattern. The expression pattern we observed was consistent with previous reports showing that NRF is expressed ubiquitously throughout the developing retina and central nervous system in zebrafish at 24 h after birth. However, at 48 h after birth, the expression of Nrf-1 was detected in the ganglion cell layer, in the neurons of the inner nuclear layer and in the optic nerve and optic tracts, but it was no longer detectable by \textit{in situ} hybridization by 72 h.\textsuperscript{47} In contrast, we found that the level of CREB1 phosphorylation was almost constant in the adult retina, and the protein could be detected in nuclei of the INL and GCL, but not in the outer nuclear layer (ONL). Furthermore, I/R surgery causes most of the damage to the INL and GCL.\textsuperscript{48} The CREB1 phosphorylation, Nrf-1 and ligase IV primarily in the INL and GCL mirrors that of \textgamma-H2AX foci. Thus, our discoveries of ligase IV transcription also support the idea that ischemia-induced damage might activate DNA NHEJ repair mediated by lithium in retinal neuroprotection.

In addition, the effects of lithium on P-CREB have a significant impact on CRE-driven gene expression in ischemia-treated cells following long-term lithium exposure. Moreover, Nrf-1 is known to control the expression of other genes.\textsuperscript{9,49,50} Thus, in neurons, regulation of DNA NHEJ repair might be one of several complex signaling pathways that respond to lithium. Thus, our results identify one mechanism through which the bioactivities of lithium might offer neural protection (Figure 8): If retinal neurocytes become ischemic and suffer genomic DNA instability, Nrf-1 expression is triggered; however, P-CREB1 is downregulated. Pretreatment with lithium upregulates the expression of P-CREB. Thus, although P-CREB1 and Nrf-1 both positively and necessarily regulate ligase IV gene expression, Nrf-1 triggers the process of neuroprotective DNA NHEJ repair mediated by lithium.

In summary, although it is known that lithium promotes neuroprotection from ischemia-induced neuronal damage, the drug’s therapeutic mechanisms have remained obscure. The present study provides substantial evidence that, to offer neuroprotection in the face of ischemia-induced damage, lithium might promote DNA NHEJ repair by upregulating ligase IV. This study should not only provide new insights into lithium-medicated neuroprotection but also might extend the application of lithium in clinical therapy.

Materials and Methods

\textbf{In vitro experiment}

Primary rat retinal neurocytes cultures and drug treatment. Retinas were dissociated enzymatically to make a suspension of single cells, essentially as described previously.\textsuperscript{11} Briefly, P1-day-old SD rats were killed by an intraperitoneal injection of Nembutal (60 mg/kg) (Sigma-Aldrich, St Louis, MO, USA). The retinas were
Figure 8 Ischemia-induced damage activates DNA NHEJ repair in the lithium-mediated retina. (1) Ischemia induces DNA damage in retinal neurocytes. (2) Ischemic damage upregulates the transcription factor Nrf-1. (3) Ischemia damage downregulates the transcription factor P-CREB1. (4) P-CREB1 and Nrf-1 positively regulate ligase IV gene expression. (6) Ligase IV binds to the breaks sites of genomic DNA in retinal neurocytes. (7) Ligase IV promotes DNA NHEJ repair and cell survival that were separated from the enucleated eyeballs were incubated for 20 min at 37 °C in a solution containing 0.125% trypsin to dissociate their cells. To yield a suspension of single cells, the tissue was then triturated sequentially through a narrow-bore Pasteur pipette in a solution of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were then seeded at a density of 1 × 10^6 cells per ml on the culture plate precoated with 0.01% poly-L-lysine for 2 h, and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. After 12 h, the cells were treated with 10 μM/ml Ara-C (Sigma) to suppress the growth of non-neurocytes. After the cells were cultured for 12 h and treated as described above, the media were replaced with complete medium (10% FBS). The cultured cells were characterized by staining with Map-2 antibody (Boster, Wuhan, China).

At 3 days after culture, the cells were incubated in the presence or absence of lithium chloride, respectively. At 24 h after incubation, the culture media were replaced with serum-free media to initiate nutrient-deprived state in ischemia in one group. For controls, the cells were cultured in full media with or without lithium chloride. The media were changed every two days.

Human retinoblastoma cells, the WERI-Rb1 cell line, rat glioma C6 cells (ATCC, Manassas, VA, USA) and the mouse retinal cell lines (RGC-5, a generous gift of Dr Xuri Li, Manassas, VA, USA) and the mouse retinal cell lines (RGC-5, a generous gift of Dr Xuri Li, Manassas, VA, USA) were cultured at a density of ~1 × 10^6 cells per ml on the culture plate precoated with 0.01% poly-L-lysine. The media were changed every two days.

**Ligase IV promoter-reporter assay.** Rat glioma C6 cells and primary rat retinal neurocytes were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) as described previously.11,12 Plasmids transfected contained 2 μg of various reporter plasmids, 2 μg of expression plasmids or pcDNA3-based vectors and 100 ng of Renilla luciferase reporter plasmid, pCMV-RL (Promega, Madison, WI, USA). The pCMV-RL plasmid encoding Renilla luciferase was included in all the samples to monitor the transfection efficiency. At 24 h post-transfection, the levels of firefly and Renilla luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase Assay System (Promega). The levels of firefly luciferase activity were normalized to Renilla luciferase activity.

**MTT assay.** Cell viability was determined by MTT assay at different time points after ischemic insult and lithium chloride treatment. Cell viability was the optical density ratio of a treated culture over an untreated control.

**Western blotting.** Western blotting assay was carried out as a standard protocol. The following antibodies were used: CREB1 (Cell Signaling Technology, Beverly, MA, USA), P-CREB1 (Cell Signaling Technology, Nrf-1 (Santa Cruz, CA, USA) and ligase IV (Santa Cruz), respectively. The membrane was incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology). GAPDH (Proteintech, Chicago, USA) served as a loading control. Protein bands were detected using an Enhanced Chemiluminescence Detection System (Millipore, Billerica, MA, USA).

**Immunofluorescence analysis of cells.** Immunofluorescence assay was carried out as standard protocol. For the assay of DNA damage, cells were fixed and analyzed for γ-H2AX foci formation by immunofluorescence with anti-phospho-H2AX ser-139 antibody (Millipore) and anti-Map-2 antibody (Boster). Images were captured using a fluorescence microscope (Carl Zeiss, Micromaging GmbH, Göttingen, Germany). The amount of γ-H2AX foci was scored in images obtained using a constant exposure time. At least 600 cells were counted.

**CHIP assay.** CHIP was performed using the CHIP Assay Kit (Upstate Cell Signaling Solutions, Lake placid, NY, USA) according to the manufacturer's instructions. The following antibodies were used: Nrf-1 (Santa Cruz) and P-CREB1 (Cell Signaling Technology), respectively, or normal IgG (Sigma-Aldrich). PCR amplification were performed using the primers spanning the ATF site on the ligase IV promoter (P-CREB1, forward, 5′-GGGTACCCTCTGTCAGTGAAAGCCA-3′; Nrf-1, forward, 5′-GGTTTATAGCCTCCTCCATGCT-3′; reverse, 5′-AAGCCACCCGCTTCCCGCC-3′), or normal IgG (Sigma-Aldrich). PCR amplification were performed using the primers spanning the ATF site on the ligase IV promoter (P-CREB1, forward, 5′-GGGTACCCTCTGTCAGTGAAAGCCA-3′; Nrf-1, forward, 5′-GGTTTATAGCCTCCTCCATGCT-3′; reverse, 5′-AAGCCACCCGCTTCCCGCC-3′), or normal IgG (Sigma-Aldrich). PCR amplification were performed using the primers spanning the ATF site on the ligase IV promoter (P-CREB1, forward, 5′-GGGTACCCTCTGTCAGTGAAAGCCA-3′; Nrf-1, forward, 5′-GGTTTATAGCCTCCTCCATGCT-3′; reverse, 5′-AAGCCACCCGCTTCCCGCC-3′).
NHEJ assay in vitro. Primary rat retinal neurons were suspended and transfected with the linearized plasmid pEGFP-N1 digested with EcoR1 using the AMAXA Electroporation System (Lonza, Levallois-Perret, France). The intact pEGFP-N1 was used as the positive control and treatment with pcDNA3.1 was used as the negative control. After 48 h, the cells were harvested and the staining fluorescence intensity was measured using a BD FACSort flow cytometer (BD Biosciences, San Jose, CA, USA). The green fluorescent cells represented the intensity levels of a light flash delivered by a photic stimulator (Ganzfeld Q400; Roland, Heidelberg, Germany) and the preamplifier bandwidth was set at 0.3–300 Hz. ERG wavelets of each rat were recorded three times and the average amplitudes of a- and b-waves were analyzed. The amplitude of the a-wave was measured as the distance from the baseline to the trough of the a-wave, whereas that of the b-wave was measured as the distance from the trough of the a-wave to the peak of the b-wave.

NHEJ assay in vivo: Preparation of plasmid/jetPEI complex. The plasmid pEGFP-N1 was digested with EcoR1 and then the concentration was adjusted with ultrapure water to 4 μg/μl. The linearized pEGFP-N1 was mixed with jetPEI (in vivo-jetPEI; Polyplus Transfection, Illkirch, France) according to the manufacturer’s instructions. In this study, the complexes were prepared as follows: the required amounts of plasmid and transfection reagent were each separately diluted in 10 μl 10% glycerol. A volume of 0.05 μl of jetPEI was used per μg of plasmid. The plasmid solution was added to the transfection reagent solution and left for 15 min at room temperature before injection. Plasmid pcDNA3.1 was used as the negative control.

Intravitreal injections: A total of 32 adult Sprague-Dawley rats weighing 250–300 g were used for intravitreal injections. Experiments were performed in two groups (16 rats for each group): the experimental group and the control group. The experimental group received subcutaneous injection of lithium chloride (Sigma) once a day, starting from 1 week before ischemia and ending on the day of tissue sampling, whereas the control group received the same amount of PBS only. Daily dose of lithium chloride was 1.0 mEq/kg. Each group was divided into two subgroups (eight rats for each subgroup): I/R experiment was carried out in one subgroup, and the other subgroup with no I/R was treated as the control. At 24 h after I/R surgery, intravitreal injections were put into execution in the right eye of all rats. A volume of 2.5 μl plasmid/jetPEI complex was used for each injection. Intravitreal injections were performed under aseptic conditions using a Hamilton syringe.

Statistical analysis. All in vitro experiments were performed in triplicate. Data are expressed as means ± SEM. The differences between mean values were evaluated with the two-tailed Student’s t-test (for two groups) and the analysis of variance (for >2 groups). All calculations and statistical tests were performed by the computer programs Microsoft Excel 2003 (Microsoft, Redmond, WA, USA) or SPSS 11.5 (SPSS, Chicago, IL, USA). P<0.05 was considered significant for all analyses.

Conflict of Interest
The authors declare no conflict of interest.

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ERG. After overnight dark adaptation, the rats were weighed and anesthetized under dim red illumination and their pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine. The rats were placed on a heating platform to maintain the body temperature at 37 °C, and local anesthetizing eye drops and hydroxypropyl methylcellulose were also applied to the corneal surface. Stainless-steel circulina record electrodes were placed on the central cornea surface. The reference electrode was placed under the tongue and the ground electrode was placed subcutaneously in the tail. Electrode impedance was checked before and after each measurement and was <5 kΩ at 25 Hz. Eyes were stimulated with 2.5 cd·s/m² intensity levels of a light flash delivered by a photic stimulator (Ganzfeld Q400; Roland, Heidelberg, Germany) and the preamplifier bandwidth was set at 0.3–300 Hz. ERG wavelets of each rat were recorded three times and the average amplitudes of a- and b-waves were analyzed. The amplitude of the a-wave was measured as the distance from the baseline to the trough of the a-wave, whereas that of the b-wave was measured as the distance from the trough of the a-wave to the peak of the b-wave.

Immunohistofluorescence assay: Retinal slides were fixed with 4% paraformaldehyde at room temperature for 20 min and subsequently incubated with 0.5% Triton X-100 for 10 min. Then, the samples were treated with a blocking solution (5% normal goat serum and 2% bovine serum albumin in PBS) for 30 min to prevent nonspecific antibody–antigen binding. γ-H2AX, ligase IV, Nrf-1, CACCAC CACACTGTGCCCA T-3 and TGGTTCCACCTCTCCA TCAG-3.

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