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Lithium reduces apoptosis and autophagy after neonatal hypoxia–ischemia

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Lithium is used in the treatment of bipolar mood disorder. Reportedly, lithium can be neuroprotective in models of adult brain ischemia. The purpose of this study was to evaluate the effects of lithium in a model of neonatal hypoxic–ischemic brain injury. Nine-day-old male rats were subjected to unilateral hypoxia–ischemia (HI) and 2 mmol/kg lithium chloride was injected i.p. immediately after the insult. Additional lithium injections, 1 mmol/kg, were administered at 24-h intervals. Pups were killed 6, 24 or 72 h after HI. Lithium reduced the infarct volume from 24.7 ± 2.9 to 13.8 ± 3.3 mm^3 (44.1%) and total tissue loss (degeneration + lack of growth) from 67.4 ± 4.4 to 38.4 ± 5.9 mm^3 (43.1%) compared with vehicle at 72 h after HI. Injury was reduced in the cortex, hippocampus, thalamus and striatum. Lithium reduced the ischemia-induced dephosphorylation of glycogen synthase kinase-3β and extracellular signal-regulated kinase, the activation of calpain and caspase-3, the mitochondrial release of cytochrome *c* and apoptosis-inducing factor, as well as autophagy. We conclude that lithium could mitigate the brain injury after HI by inhibiting neuronal apoptosis. The lithium doses used were in the same range as those used in bipolar patients, suggesting that lithium might be safely used for the avoidance of neonatal brain injury.

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Lithium is widely used in the treatment of bipolar disorder. Recent studies have shown unexpected neuroprotective effects in a variety of animal models of neurodegenerative diseases.[1–3] The mechanisms underlying lithium-mediated neuroprotection are complex and may include activation of the phosphatidylinositol 3-kinase/Akt[4] pathway, activation of the extracellular signal-regulated kinase (ERK) cell survival pathway,[5] in addition to yet poorly understood pathways that promote neuronal survival and proliferation.[6,7] Lithium treatment may reduce caspase-3[8] and calpain[9] activation, as a correlate of neuroprotection. Lithium has been shown to inhibit glycogen synthase kinase-3β (GSK-3β) activity through direct or indirect mechanisms.[4,10] GSK-3β is generally considered to have a proapoptotic role in thus for that its inhibition confers cytoprotection.[11] Taken together, these findings provide a strong rationale for the exploration of lithium as a potential treatment of neurodegenerative diseases.[12]

Lithium has a potent neuroprotective effect in adult brain ischemia-reperfusion injury.[3,8] However, no information is available on possible effects of lithium in neonatal brain injury. At difference with neurons contained in the adult brain, neurons from newborn rodents have a high propensity to undergo apoptotic cell death, presumably because they still express high levels of caspases.[13,14] Given the peculiar importance of apoptotic cell death in the immature brain, one may speculate that lithium could be particularly neuroprotective in this setting,[13,15] a hypothesis that we examined in this study.

Perinatal asphyxia-induced brain injury is one of the most common causes of morbidity and mortality, both in term and preterm neonates, accounting for 23% of neonatal deaths globally.[16] Survivors of perinatal asphyxia suffer long-term neurological disability and impairment with major socio-economic implications. Hypoxic–ischemic encephalopathy (HIE) remains a major cause of acute perinatal brain injury, ultimately leading to neurological dysfunctions such as cerebral palsy, mental retardation and epilepsy. Although many neuroprotective strategies have seemed promising in animal models, most of them were not feasible or effective in human newborns. There are very few randomized controlled clinical trials that have shown improved outcomes for term neonates. One method shown to have beneficial effects is hypothermia. Cooling the brains of term infants with moderate HIE reduced the rate of disability if the treatment was initiated within 6 h.[17] Another, recently published trial showed that a similar reduction of the rate of disability could be obtained by repeated injections of low-dose human erythropoietin.[18] Thus, there is an urgent, unmet need to develop new brain-salvaging strategies that may be used either alone or in combination with hypothermia and/or erythropoietin. In this

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Abbreviations: AIF, apoptosis-inducing factor; CA, cornu ammonis; Cyt *c*, Cytochrome *c*; ERK1/2, extracellular signal-regulated kinase (ERK1/2); FBDP, fodrin breakdown product; GSK-3β, glycogen synthase kinase-3β; HI, hypoxia–ischemia; HIE, hypoxic–ischemic encephalopathy; LC3, microtubule-associated protein 1, light chain 3
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study, we investigated the potential neuroprotective effects of post-insult administration of lithium in neonatal rat hypoxia-ischemia (HI) brain injury.

Results

Lithium reduced HI brain injury. At 72 h after HI, the histologically detectable brain injury encompassed areas in the cortex, hippocampus, striatum and thalamus (Figure 1a). Lithium treatment that was initiated shortly after HI was highly efficient in reducing brain injury. The infarct volume was $24.7 \pm 2.9\,mm^3$ in the vehicle group and $13.8 \pm 3.3\,mm^3$ in the lithium-treated group, which corresponds to a 44.1% decrease after lithium treatment ($P = 0.016$) (Figure 1b). The total tissue loss was $67.4 \pm 4.4\,mm^3$ in the vehicle group and $38.4 \pm 5.9\,mm^3$ in the lithium group, corresponding to a 43.1% decrease ($P = 0.0003$) (Figure 1c). Moreover, lithium treatment reduced the pathological scores in all affected brain areas including the cortex, hippocampus, striatum and thalamus (Figure 1d).

Lithium prevents the acute dephosphorylation of GSK-3β and ERK1/2 after HI. GSK-3β is normally inhibited by Akt-mediated phosphorylation of the serine-9 residue. Phospho-GSK-3β-Ser9 (the inactive, protective form of GSK-3β) was decreased 6 h after HI in the injured, ipsilateral hemisphere, and this dephosphorylation was largely prevented by lithium treatment (Figures 2a and b). Phospho-GSK-3β-Ser9 further decreased in both hemispheres 24 h after HI, and at this time point there was no longer any difference between the treatment groups (Figure 2a, lower panels). ERK1/2 activation (phosphorylation) decreased by approximately 80% in the ipsilateral hemisphere 6 h after HI compared with control or the contralateral hemisphere (Figure 2c). Again, this dephosphorylation was partially prevented by lithium treatment, such that the amount of P-ERK1/2 almost doubled (and actually increased by 87%, $P = 0.0493$, and 81.4%, $P = 0.0487$, for P-ERK1 and P-ERK2, respectively) in the ipsilateral hemisphere (Figures 2d and e). As in the case of GSK-3β, both the ipsi- and the contralateral hemispheres showed progressive loss of phosphorylated ERK1/2 during recovery after HI, and at 24 h after HI there was only 10% P-ERK1/2 left. At this late time point, there were no differences between the two hemispheres or between the two treatment groups (Figures 2c–e).

Lithium inhibits calpain and caspase activation after HI. We have previously shown that calpains and caspase-3 are activated after HI in the immature brain.[13] Caspase-3 activation is a hallmark of apoptosis, whereas

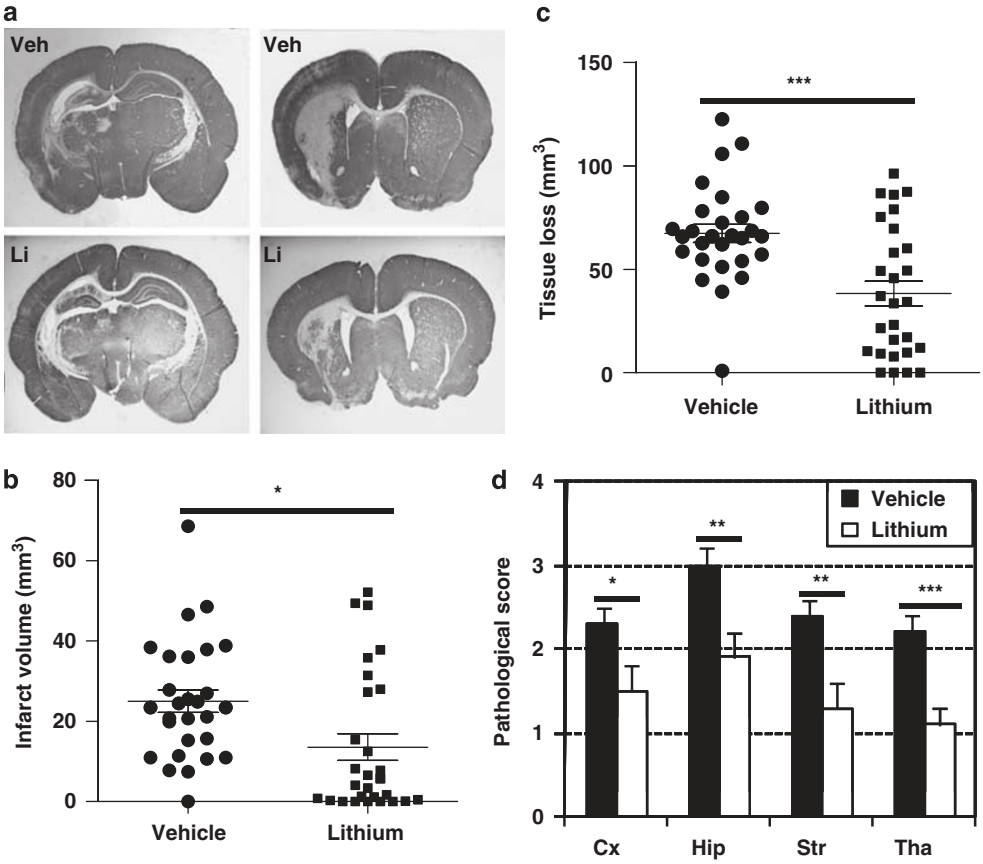

Figure 1 Lithium treatment reduced HI brain injury. (**a**) Representative MAP2 stainings on the level of dorsal hippocampus (left panels) and striatum (right panels) 72 h after HI of vehicle- (upper panels) and lithium-treated mice (lower panels). (**b**) The infarct volume was reduced by 44.1% in lithium-treated mice ($n = 28$) as compared with vehicle-treated mice ($n = 28$). (**c**) The volume of total tissue loss was reduced by 43.1% after lithium treatment. (**d**) Neuropathological scores showed less injury in all the observed brain regions after lithium treatment. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

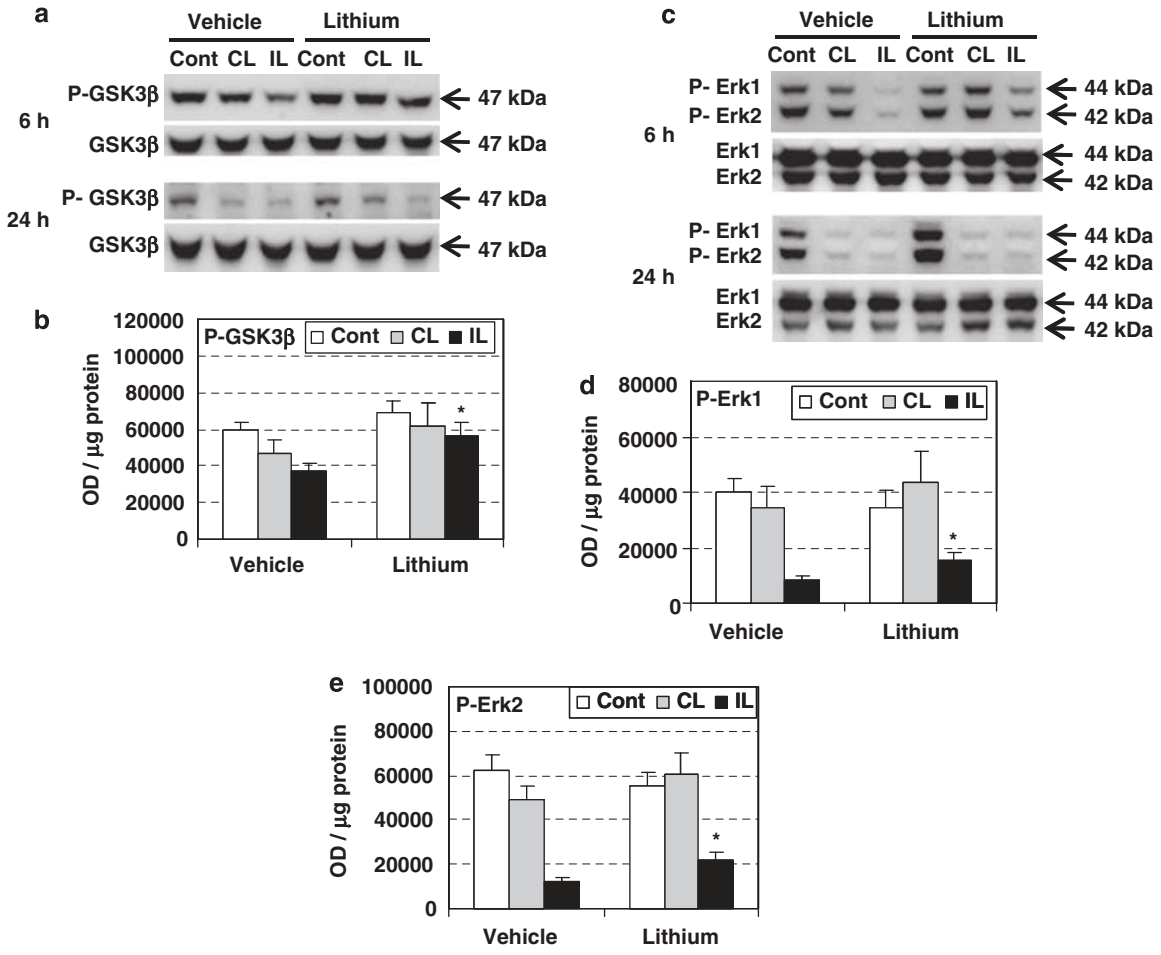

Figure 2 Lithium prevented GSK-3β and ERK dephosphorylation. (**a**) Representative P-GSK-3β and GSK-3β imunoblots 6 h (upper panel) and 24 h (lower panel) after HI. (**b**) Quantification of P-GSK-3β 6 h after HI showed that lithium prevented the loss in the ipsilateral hemisphere. (**c**) Representative P-ERK1/2 and ERK1/2 imunoblots 6 h (upper panel) and 24 h (lower panel) after HI. (**d** and **e**) Quantification of P-ERK1 (figure **d**) and P-Erk2 (figure **e**) 6 h after HI showed partial prevention of ischemia-induced loss in the ipsilateral hemisphere after lithium treatment ($n = 7$ for HI 24 h, $n = 6$ for all other groups). *$P < 0.05$ compared with the vehicle group. Cont, control; CL, contralateral; IL, ipsilateral

calpain activation may reflect both necrosis and apoptosis.[19] In this study, we determined the abundance of the 150 kDa fodrin breakdown product (FBDP), to indirectly assess calpain activation.[19,20] FBDP-positive cells were increased in the ipsilateral hemisphere after HI, and lithium treatment decreased the number of FBDP-positive cells 6 h after HI in the cortex (Figure 3b) and 24 h after HI in the hippocampus (Figure 3c). The intact 240 kDa fodrin was cleaved after HI, revealing the calpain-generated 145/150 kDa products and the caspase-3-generated 120 kDa product, all of which were less abundant in the lithium-treated brains (Figure 3d). Caspase-3 activity increased 30-fold 24 h after HI, and this increase was completely abolished after lithium treatment (Figure 4a). The number of active caspase-3-positive cells increased gradually during reperfusion after HI (Figure 4b), and lithium treatment decreased the number of active caspase-3-positive cells in the hippocampus 24 h after HI (Figure 4c). This was confirmed also by immunoblotting, showing that lithium treatment prevented processing of the 32 kDa caspase-3 proform into the intermediate 29 kDa and active 17 kDa fragments[19] (Figure 4d). Furthermore, the endogenous caspase inhibitor, XIAP, was upregulated after lithium treatment (Figure 4d).

Lithium inhibits mitochondrial release of apoptosis-related proteins. Cytochrome *c* (Cyt *c*) is located in the mitochondrial intermembrane space under normal conditions, as indicated by a weak punctate cytoplasmic immunostaining[21] (Figure 5a). In damaged areas, Cyt *c* was released from mitochondria to the cytoplasm, producing an intense neuronal, cytoplasmic staining (Figure 5a). Lithium treatment decreased the number of Cyt *c*-positive cells in the ipsilateral hemisphere 24 h after HI in both cortex and hippocampus (Figures 5b and c). Immunoblotting confirmed that the amount of of Cyt *c* that was associated with the mitochondrial fractions was enhanced after lithium treatment. This effect was most pronounced 24 h after HI (Figure 5e), at the same time when the reduction in caspase-3 activation (shown above) is maximal. Apoptosis-inducing factor (AIF) is another protein that is usually located in the intermembrane space of mitochondria and that translocates to the nucleus from injured cells (Figure 6a). Lithium treatment decreased

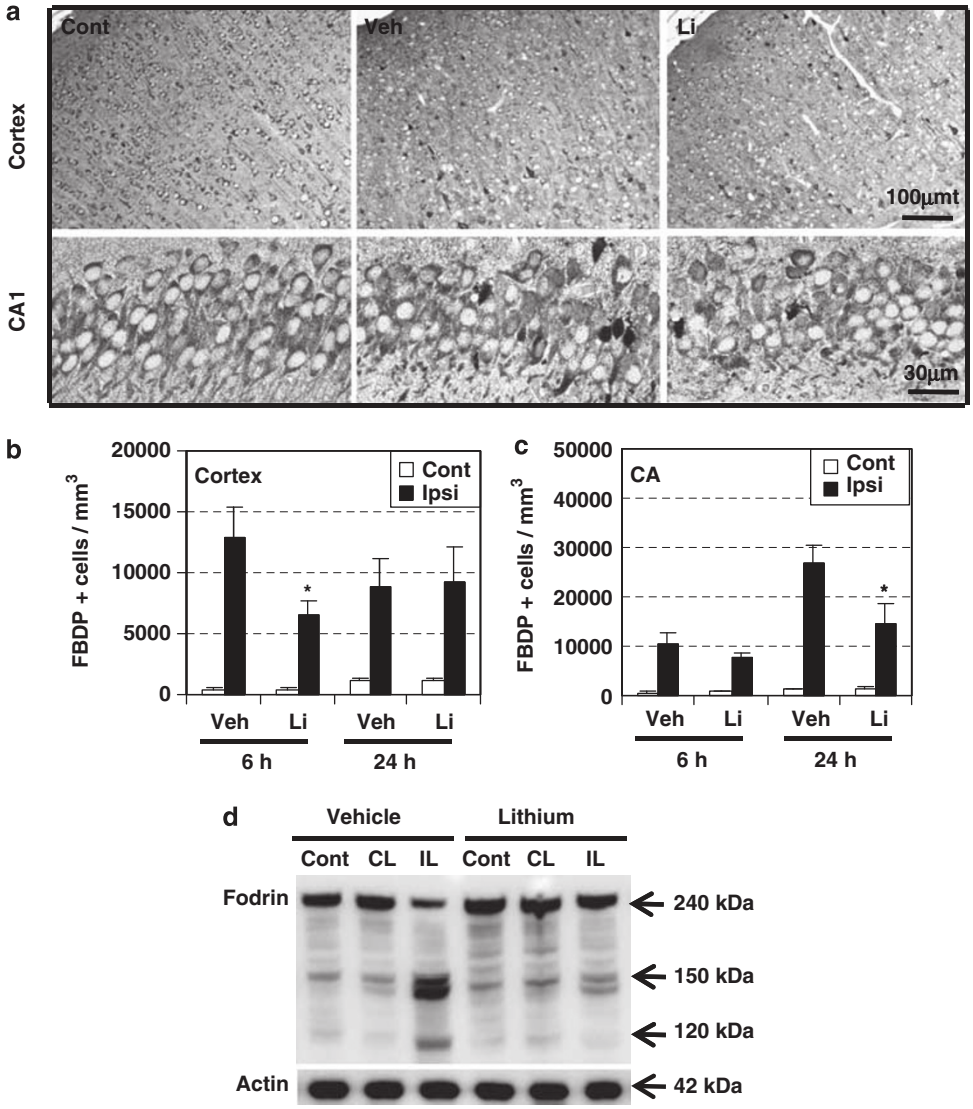

Figure 3 Calpain activation after HI. (**a**) Representative FBDP stainings in the cortex (upper panel) and CA1 of the hippocampus (lower panel). (**b**) Quantification of FBDP-positive cells in the cortex showed significantly fewer cells after lithium treatment 6 h after HI. (**c**) Quantification of FBDP-positive cells in the hippocampus showed significantly fewer cells after lithium treatment 24 h after HI ($n=6$ for HI 6 h, $n=8$ for HI 24 h). (**d**) A representative fodrin immunoblot of homogenates showing that the 240 kDa fodrin was cleaved in the ipsilateral hemisphere, producing 150 and 120 kDa breakdown products 24 h after HI, and that lithium treatment prevented fodrin breakdown. *$P<0.05$ compared with the vehicle group ($n=7$ for HI 24 h, $n=6$ for all other groups). Cont, control; CL, contralateral; IL, ipsilateral

the number of AIF-positive nuclei 24 h after HI in the hippocampus (Figure 6b), and immunoblotting confirmed that lithium inhibited the AIF release from the mitochondrial fraction (Figure 6c).

Lithium inhibits post-ischemic autophagy. LC3 immunostaining revealed intense, punctate staining in the cytoplasm of neurons, particularly in injured areas (Figure 7a). The number of LC3-positive cells was lower in lithium-treated animals, an effect that was most pronounced 72 h after HI (Figures 7a and b). Immunoblots confirmed increased autophagy in the ipsilateral hemisphere 72 h after HI, as judged by increased levels of the 14 kDa LC3-II, and this biochemical sign of increased autophagy was reduced by lithium treatment (Figure 7c).

Discussion

Lithium was discovered as a new element in 1817 by the Swedish chemist Johan Arfwedson, and lithium has been used for the treatment of mania from the 1870s onward. In addition to the well-documented mood-stabilizing effects of lithium in bipolar patients, recent *in vitro* and *in vivo* studies have implicated lithium as a neuroprotective agent.[22] Putative mechanisms include inhibition of GSK-3β, stimulation of heat shock protein-70, inhibition of Ca^{2+} influx through NMDA receptors, as well as activation of the ERK signaling pathway.[23] However, these postulated mechanisms are based on evidence generated in the adult nervous system. Cell death cascades after ischemic insults are developmentally regulated and apoptotic mechanisms seem to be particularly important in the immature brain.[13,15] To the best of our

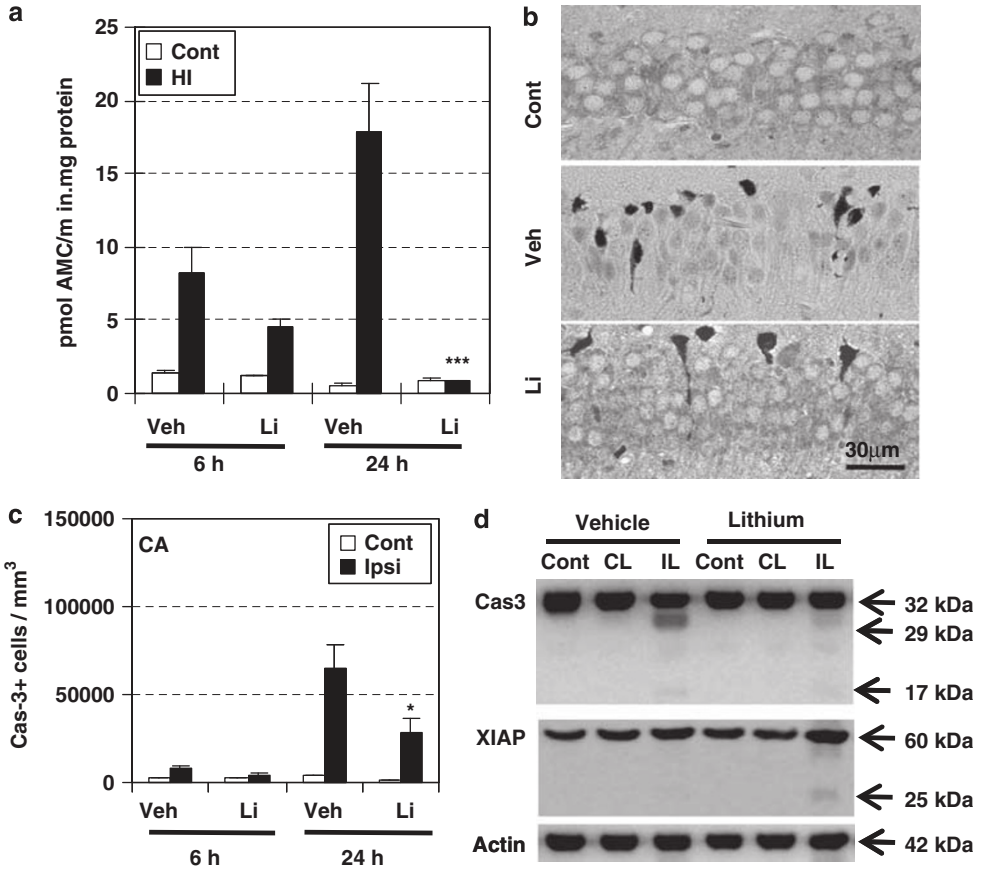

Figure 4 Lithium prevented caspase-3 activation after HI. (**a**) Caspase-3 activity (DEVD cleavage) was measured 6 and 24 h post-HI in both vehicle- (Veh) and lithium- (Li) treated rats ($n=7$ for HI 24 h, $n=6$ for all other groups). Caspase-3 activation was abolished 24 h after HI by lithium treatment. (**b**) Representative immunostainings for activated caspase-3 in CA of the hippocampus in normal control (Cont), and Veh- or Li-treated rats 24 h after HI. (**c**) Quantification of active caspase-3-positive cells in CA of the hippocampus showed significant reduction in the lithium-treated rats 24 h after HI ($n=6$ for HI 6 h, $n=8$ for HI 24 h). (**d**) Representative caspase-3, XIAP and actin immunoblots of normal control (Cont), contralateral (CL) and ipsilateral (IL) hemispheres 24 h after HI. The 32 kDa proform was cleaved, and produced 29 and 17 kDa fragments after HI (upper panel). XIAP immunoblotting showed lithium-induced upregulation in the ipsilateral hemisphere 24 h after HI (middle panel). The lower panel (actin) indicates equal protein loading ($n=7$ for HI 24 h, $n=6$ for all other groups). *$P<0.05$, ***$P<0.001$ compared with the Veh-treated HI group

knowledge, there are no published studies showing neuroprotective effects of lithium in a neonatal brain injury paradigm.

GSK-3, originally identified as a regulator of glycogen metabolism, is now known as a multifaceted enzyme affecting a diverse range of biological functions, including gene expression, cellular architecture and apoptosis. In particular, GSK-3β is well known to have critical roles in oxidative stress-induced neuronal cell death by enhancing the expression of pro-apoptotic proteins and by inhibiting the activity of anti-apoptotic proteins. Lithium inhibits GSK-3β activity by direct binding to the magnesium-sensitive active site of the enzyme[24] and also indirectly by inducing the phosphorylation of GSK-3β-Ser9 by other kinases.[4,25] Emerging evidence supports the idea that GSK-3β inhibition is involved in the neuroprotective effects of lithium.[1,26,27]

An earlier study showed that P-Akt and P-GSK-3β immunoreactivity was lost in the immature brain after HI and that IGF-I treatment increased P-Akt, prevented P-GSK-3β downregulation and afforded neuroprotection.[28] GSK-3β is inhibited by phosphorylation at Ser9 through Akt. GSK-3β may induce caspase activation either through the intrinsic pathway or through downregulation of heat shock proteins.[29] On the basis of these previous studies, we speculate that lithium-induced suppression of GSK-3β may account for its anti-apoptotic and cytoprotective effects. Similarly, activation of ERK1/2 occurred early in neurons after HI in the neonatal brain[30] and has been coupled to protective mechanisms,[26] including lithium-mediated neuroprotection.[5] At this stage, it is difficult to assess the relative contribution of lithium effects on GSK-3ß and ERK1/2 with regard to neonatal neuroprotection.

Reduced fodrin breakdown indicates that calpain activity was decreased after lithium treatment.[21] However, there is no evidence that lithium inhibits calpain activity directly.[2] Therefore, inhibition of fodrin breakdown by lithium is probably secondary to the general cytoprotective action of lithium and/or to caspase inhibition,[19] as there is crosstalk between calpains and caspase-3.[19] Apoptotic cell death has a prominent role in the evolution of HI brain injury in neonates.[13] Our results indicate that lithium treatment inhibited mitochondrial release of both Cyt *c* and AIF, and subsequent apoptotic cell death. GSK-3β activation has been shown to be critical for the mitochondrial release of both Cyt *c* and AIF,[31] supporting the conjecture that lithium inhibits mitochondrial outer

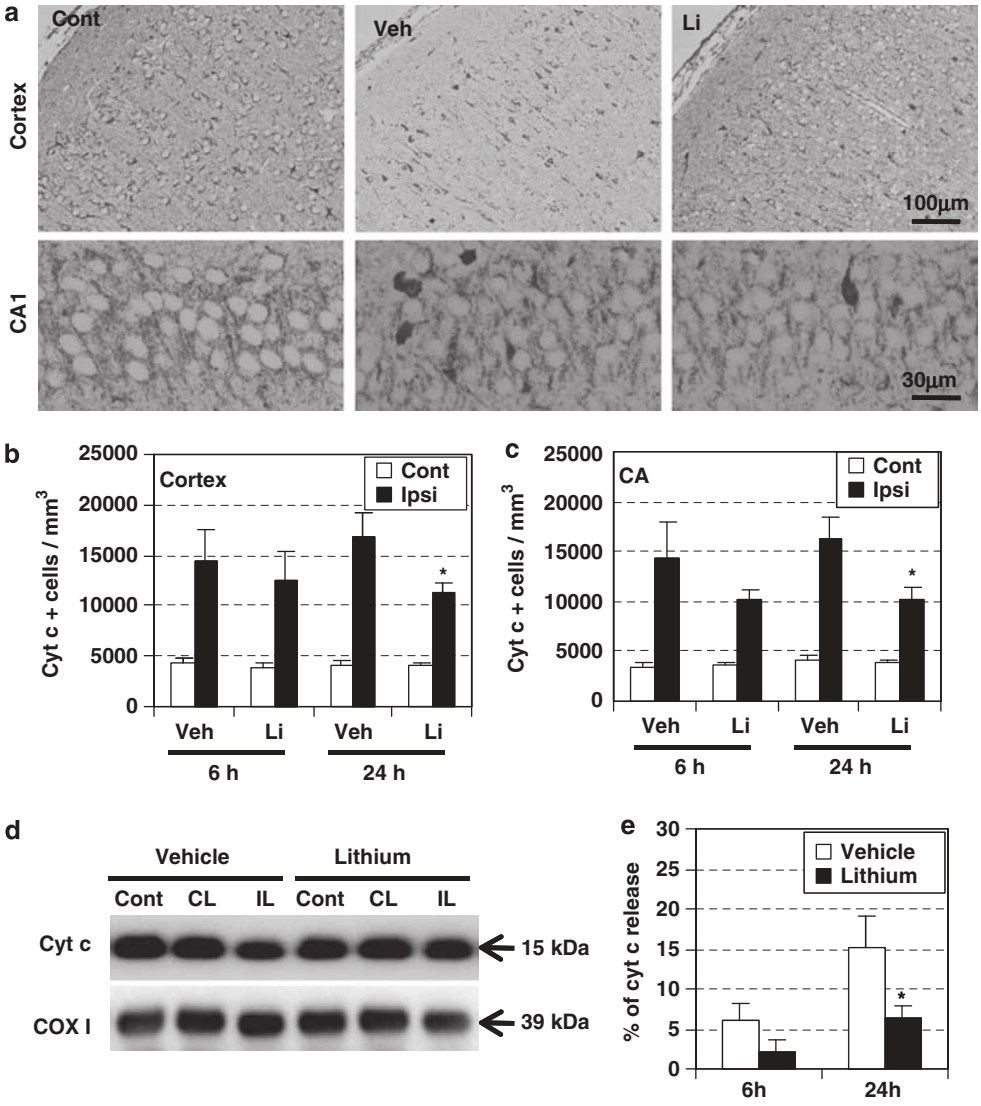

Figure 5 Lithium prevented Cyt *c* release after HI. (**a**) Representative Cyt *c* stainings in the cortex (upper panel) and CA of the hippocampus (lower panel). (**b**) The number of Cyt *c*-positive cells in the cortex was lower after lithium treatment 24 h after HI. (**c**) The number of Cyt *c*-positive cells in the CA of the hippocampus was also lower 24 h after HI after lithium treatment ($n = 6$ for HI 6 h, $n = 8$ for HI 24 h). (**d**) A representative Cyt *c* immunoblot of mitochondrial fractions. (**e**) Quantification showed that Cyt *c* release from mitochondrial fractions 24 h after HI was reduced from 15 to 6% after lithium treatment ($n = 7$ for HI 24 h, $n = 6$ for all other groups). $^*P < 0.05$ compared with the vehicle group

membrane permeabilization by virtue of its action on GSK-3β. Other studies have found that lithium treatment increased the expression of Bcl-2 or heat shock protein and reduced the levels of the pro-apoptotic proteins p53 and Bax.[1,32] Similarly, we found that lithium upregulated the expression of the endogenous caspase inhibitor XIAP. Together, these data suggest that lithium-mediated neuroprotection may involve multiple anti-apoptotic mechanisms.[33]

Autophagy, a lysosomal pathway for intracellular bulk degradation of cytoplasmic macromolecules and organelles, has a key role to maintain cellular homeostasis and survival. In this study, we show that autophagy was induced in the neonatal brain after HI, as indicated by LC3 immunoblotting.[13,34] However, it is still debated whether enhanced autophagy is involved in cell death[35] or whether it represents a rescue mechanism.[36] Indeed, it has been suggested that autophagy can have either deleterious or protective effects, depending on the specific cellular context and the stage of the pathological process.[37] One *in vitro* study showed that lithium could induce autophagy,[38] but our current findings rather indicate that lithium prevented autophagy during late recovery after HI. It remains elusive whether this is secondary to the lithium-mediated tissue protection resulting in less cellular damage and hence less intracellular debris to be processed by autophagy, or whether lithium directly inhibits autophagy and thereby contributes to tissue protection.

Lithium is used in the treatment of bipolar affective disorder since decades and has been used extensively off-label for several other neuropsychiatric disorders. However, it has not been approved, nor even suggested for fetal or neonatal neuroprotection. The doses used in this study are in the same range as those used for adult humans,[39] and it is encouraging

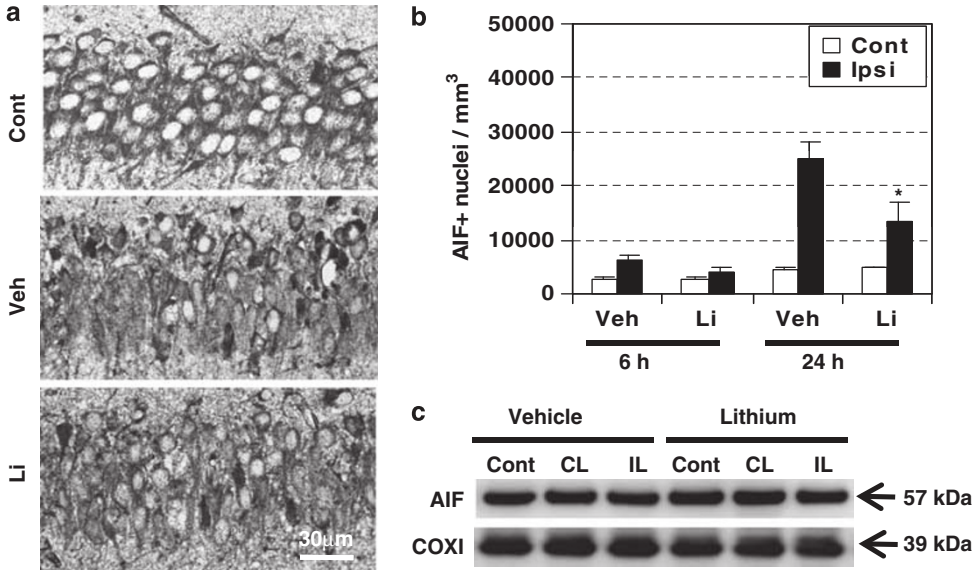

Figure 6 AIF translocation 24 h after HI. (**a**) Representative AIF stainings from CA of the hippocampus. (**b**) Quantification revealed fewer AIF-positive nuclei in the hippocampus after lithium treatment 24 h after HI ($n=6$ for HI 6 h, $n=8$ for HI 24 h). (**c**) A representative AIF immunoblot of mitochondrial fractions. $^*P<0.05$ compared with the vehicle group ($n=7$ for HI 24 h, $n=6$ for all other groups)

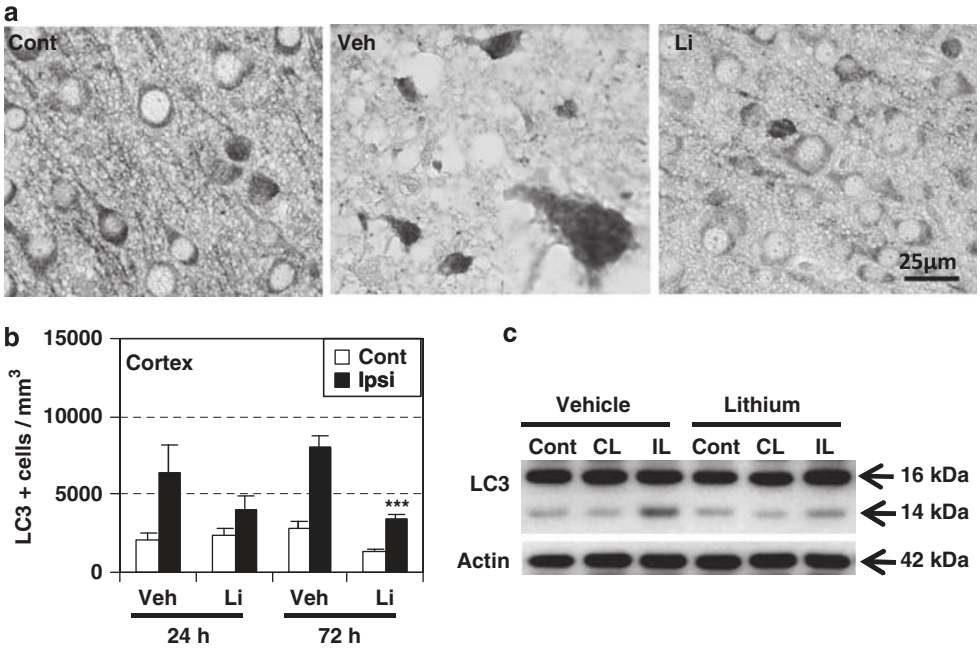

Figure 7 LC3 immunostaining after HI. (**a**) Representative LC3 stainings from the cortex. (**b**) Quantification revealed fewer LC3-positive cells in the cortex after lithium treatment 72 h after HI ($n=8$ for 24 h, $n=10$ for 72 h). (**c**) A representative LC3 immunoblot of homogenates 72 h after HI ($n=7$ per group)

that a single daily administration was sufficient to confer neuroprotection. On the basis of these data, we conclude that a further preclinical exploration of lithium for the treatment or prevention of neonatal brain damage is warranted.

Materials and Methods

hypoxia–ischemia. Postnatal day 9 (P9) male Wistar rat pups were purchased from B&K Universal AB (Sollentuna, Sweden). The rats were randomly assigned to either lithium chloride or vehicle (saline) treatment. Animals were anesthetized with isoflurane (5% for induction, 1.5–2.0% for maintenance) in a mixture of nitrous oxide and oxygen (1 : 1), the duration of anesthesia was <5 min. The left common carotid artery was cut between double ligatures of prolene sutures (6.0). After the surgical procedure, the wounds were infiltrated with lidocaine for local analgesia. The pups were returned to their cages for 1 h and then placed in a chamber perfused with a humidified gas mixture (7.7% oxygen in nitrogen) for 50 min at 36 °C. Following hypoxic exposure, the pups were returned to their cages. Control pups were injected either with lithium chloride or vehicle but were not

subjected to HI. All animal experimentation was approved by the Gothenburg Committee of the Swedish Animal Welfare Agency (application no. 145–2008).

Lithium administration. Lithium chloride (Aldrich, St. Louis, MO, USA) was dissolved in normal saline and injected at a dose of 2 mmol/kg intraperitoneally immediately after HI, followed by 1 mmol/kg injections at 24-h intervals. Animals surviving 6 h or 24 h after HI received one injection. Animals surviving 72 h after HI received three injections.

Immunohistochemistry. The animals were deeply anesthetized with phenobarbital and perfusion-fixed with 5% formaldehyde in 0.1 M PBS, followed by immersion fixation in the same fixative for 24 h at 4 °C. After dehydration with graded ethanol and xylene, the brains were paraffin embedded, serial cut in 5 μm coronal sections and mounted on glass slides. Every 100th section was stained for MAP-2. On the hippocampus level, every 50th section was stained. The sections were stained for the following cell death-related markers: AIF, active caspase-3, Cyt *c*, microtubule-associated protein 1, light chain 3 (LC3) and FBDP, as described earlier.[13] Sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the sections in 10 mM boiling sodium citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked for 30 min with 4% goat or horse serum (depending on the species used to raise the secondary antibody) in PBS. Rabbit anti-active caspase-3 (1 : 100, BD Pharmingen, Stockholm, Sweden), anti-FBDP (1 : 50), anti-LC3 (1 : 300, #2775, Cell Signaling, Danvers, MA, USA), goat anti-AIF (1 : 100, 2 μg/ml, sc-9416, Santa Cruz, Santa Cruz, CA, USA) or mouse anti-Cyt *c* (1 : 500, BD Pharmingen) were incubated for 60 min at room temperature, followed by the appropriate, biotinylated goat anti-rabbit (1 : 150, for active caspase-3, FBDP and LC3), biotinylated horse anti-goat (1 : 200, for AIF) or biotinglated horse anti-mouse (1 : 150) secondary antibody for 60 min at room temperature. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. Visualization was performed using Vectastain ABC Elite with 0.5 mg/ml 3,3′-diaminobenzidine enhanced with 15 mg/ml ammonium nickel sulfate, 2 mg/ml β-D glucose, 0.4 mg/ml ammonium chloride and 0.01 mg/ml β-glucose oxidase (all from Sigma, Stockholm, Sweden). The numbers of positive cells were counted on every 50th section in the cortex and the whole cornu ammonis (CA) of hippocampus and at least six sections were counted. The positive cells in the cortex were counted in the border zone and three high magnification visual fields (0.196 mm^2) were counted in each section. The positive cells in the CA were counted using unbiased stereological counting techniques (StereoInvestigator, MicroBrightField Inc., Magdeburg, Germany). The cell density was calculated from the sum of all cells counted divided by the counting volume.

Injury evaluation. Brain injury 72 h after HI was evaluated using infarct volume, total tissue loss and neuropathological scoring. The MAP2-positive and -negative areas in each section were measured using Micro Image (Olympus, Tokyo, Japan). The infarct volume was calculated from the MAP2-negative areas according to Cavalieri principle, using the following formula: $V = \sum A \times P \times T$, where $V =$ total volume, $\sum A =$ sum of area measurement, $P =$ the inverse of the sampling fraction and $T =$ the section thickness. The total tissue loss was calculated as the MAP2-positive volume in the contralateral hemisphere minus the MAP2-positive volume in the ipsilateral hemisphere. The neuropathological score for the cortex, hippocampus, striatum and thalamus was assessed as described previously.[13] Briefly, serial sections from each brain were stained for MAP2 and scored by an observer blinded to the treatment of the animals. The cortical injury was graded from 0 to 4, 0 being no observable injury and 4 indicating confluent infraction encompassing most of the cerebral cortex. The damage in the hippocampus, striatum and thalamus was assessed both with respect to hypotrophy (0–3) and observable cell injury/infarction (0–3) resulting in a neuropathological score for each brain region (0–6).

Western blotting. Animals were killed by decapitation 6, 24 or 72 h after HI. Control animals were killed at the same time as HI pups after injection. The brains were rapidly dissected out on a bed of ice. The parietal cortex (including the hippocampus) was dissected out from each hemisphere and ice-cold isolation buffer was added (15 mM Tris-HCl, pH 7.6, 320 mM sucrose, 1 mM DTT, 1 mM $MgCl_2$, 3 mM EDTA-K, 0.5% protease inhibitor cocktail (P8340; Sigma), 1% phosphatase inhibitor cocktail 1 (P2850, Sigma), 1% phosphatase inhibitor cocktail 2 (P5726, Sigma) and 2.5 μM cyclosporin A). Homogenization was performed gently by hand in a 2 ml glass–glass homogenizer (Merck Euro Lab, Dorset, England) using, sequentially, two different pestles with a total clearance of 0.12 mm and 0.05 mm, respectively (10 strokes each). The homogenates were centrifuged at 800 $\times$ *g* for 10 min at 4 °C. The pellets were washed in homogenizing buffer and re-centrifuged at 800 $\times$ *g* for 15 min at 4 °C, producing a crude nuclear pellet (P1). The supernatant from the first centrifugation was further centrifuged at 9200 $\times$ *g* for 15 min at 4 °C, producing a mitochondrial and synaptosomal fraction in the pellet (P2) and a crude cytosolic fraction in the supernatant (S2). The pellet fractions were washed in homogenizing buffer and centrifuged at 9200 $\times$ *g* for 15 min. The protein concentrations were determined according to Whitaker and Granum,[40] adapted for microplates. Samples of 65 μl were mixed with 25 μl NuPAGE LDS 4 $\times$ sample buffer and 10 μl reducing agent and heated (70 °C) for 10 min. Individual samples were run on 4–12% NuPAGE Bis-Tris gels (Novex, San Diego, CA, USA) and transferred to reinforced nitrocellulose membranes. After blocking with 30 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20 containing 5% fat-free milk powder for 1 h at room temperature, the membranes were incubated with primary antibodies: goat anti-AIF (sc-9416, 0.2 μg/ml, Santa Cruz), rabit anti-caspase-3 (H-277, 1 : 1000, Santa Cruz), mouse anti-Cyt *c* (1 : 500, clone 7H8.2C12, Pharmingen, San Diego, CA, USA), rabbit anti-actin (1 : 200, A2066, Sigma), mouse anti-fodrin (0.2 μg/ml, clone AA6, BIOMOL, Plymouth Meeting, PA, USA), anti-OxPhos Complex I 39 kDa subunit (0.5 μg/ml, p39, clone 20C11, Molecular Probes, Eugene, OR, USA), mouse anti-XIAP (clone 28, 1 : 250, BD Bioscience, Stockholm, Sweden), rabbit anti-LC3 (1 : 1000, Cell Signaling, #2775), mouse anti-GSK-3β (1 : 1000, sc-81462, Santa Cruz), rabbit anti-phospho-GSK-3β (Ser9) (1 : 1000, #9323, Cell Signaling), rabbit anti-ERK1/2 (1 μg/ml, AB3053, Chemicon, Temecula, CA, USA) and rabbit anti-phospho-ERK (Thr202/Tyr204) (1 : 1000, #9101, Cell Signaling), at room temperature for 60 min. After washing, the membranes were incubated with a peroxidase-labeled secondary antibody for 30 min at room temperature (goat anti-rabbit, 1 : 2000, horse anti-goat, 1 : 2000 or horse anti-mouse 1 : 4000). Immunoreactive species were visualized using the Super Signal West Dura substrate (Pierce, Rockford, IL, USA) and a LAS 3000 cooled CCD camera (Fujifilm, Tokyo, Japan).

Caspase activity assay. The protein concentrations were determined as above. Samples of homogenate (40 μl) were mixed with 60 μl of extraction buffer as described earlier.[20] Cleavage of Ac-DEVD-AMC (for caspase-3/7 activity, Peptide Institute, Osaka, Japan), was measured with an excitation wavelength of 380 nm and an emission wavelength of 460 nm, and expressed as pmol AMC released per mg protein and minute.

Statistics. All data were expressed as mean $\pm$ S.E.M. Student's *t*-test was used when comparing injury scores, tissue loss or the numbers of immunopositive cells between two different groups. ANOVA with Fisher's *post hoc* test was used when comparing more than two groups. Significance level was assigned at $P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

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