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ORIGINAL RESEARCH ARTICLE

Short-term lithium treatment promotes neuronal survival and proliferation in rat striatum infused with quinolinic acid, an excitotoxic model of Huntington's disease

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We assessed the ability of lithium to reduce neurodegeneration and to stimulate cell proliferation in a rat model of Huntington's disease in which quinolinic acid (QA) was unilaterally infused into the striatum. LiCl (0.5–3.0 mEq/kg) was injected subcutaneously 24 h before and 1 h after QA infusion. At 7 days after QA injection, lithium significantly diminished the loss of neurons immunostained for *Neu*ronal *N*uclei (NeuN) in the injured striatum, but failed to prevent the reduction of NADPH-diaphorase-positive striatal interneurons. Lithium also reduced the number of neurons showing DNA damage or activated caspase-3. This neuroprotection was associated with an upregulation of Bcl-2 protein levels in the striatal tissue and an increase in the number and density of Bcl-2 immunostaining in striatal neurons. Bromodeoxyuridinie (BrdU) labeling in the lithium-treated injured striatum revealed the presence of large numbers of proliferating cells near the QA-injection site, with a reduction of BrdU-labeled cells in the subventricular zone (SVZ). All BrdU-labeled cells in the SVZ and the majority of BrdU-labeled cells near the QA-injection site were negative for either NeuN or glial fibrillary acidic protein (GFAP), suggesting that they are undifferentiated progenitor cells. However, a small number of BrdU-positive cells found in the QA-injected and lithium-treated striatum site were positive for either NeuN or GFAP. Our results suggest that lithium is neuroprotective in the QA-injection model of Huntington's disease not only due to its ability to inhibit apoptosis but also because it can stimulate neuronal and astroglial progenitor proliferation in the QA-injected striatum or their migration from the SVZ.
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Huntington's disease (HD), a devastating neurodegenerative disorder with a phenotype of hyperkinetic involuntary movement, psychosis and dementia, involves a selective loss of neurons in the striatum and cerebral cortex.[1] The disease-causing mutation is an expansion of CAG repeats in the gene encoding for huntingtin.[2] One of the animal models of HD is the excitotoxicity model in which an excitotoxin, such as quinolinic acid (QA), is infused into the striatum.[1] QA is a metabolite of tryptophan in the kynurenine pathway and an endogenous agonist of *N*-methyl-D-aspartate (NMDA) receptors.[3] Its infusion into the rat striatum results in the death of medium-sized spiny neurons and produces many neuroanatomical and neurochemical manifestations of HD.[4–7] Neuromorphological and neurochemical abnormalities, and behavioral changes similar to those found in HD patients have also been observed in primates injected with QA into the striatum.[8,9] An increased sensitivity to QA-induced excitotoxicity was observed in the striata of some symptomatic transgenic mice carrying expanded CAG repeats in the huntingtin gene.[10,11] The excitotoxicity hypothesis of HD has been corroborated by an imaging study showing disordered glutamate metabolism in the brain of HD patients,[12] and clinical trials showing reduced chorea in HD patients treated with an NMDA receptor antagonist amantadine.[13]

We have found that lithium, the primary drug used to treat bipolar mood disorder, has robust neuroprotective effects against excitotoxicity mediated by NMDA receptors in cultured brain neurons,[14–17] and brain damage induced by focal ischemia in rats.[18,19] Molecular and cellular studies suggest that neuroprotective mechanisms of lithium are complex and may include inactivation of NMDA receptors and changes in the expression of a number of proteins involved in apoptosis and cytoprotection.[20] Inhibition of glycogen synthase kinase-3beta also likely contributes to lithium neuroprotection.[21] Previously, we have found that QA-induced striatal injury is markedly attenuated by long-term (16-day) pretreatment with subcutaneous injections of lithium.[22] In addition to the

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antiapoptotic actions, lithium facilitates neurogenesis in rat hippocampus,[23] and stimulates progenitor cell proliferation in brain neuronal cultures.[24,25] The present study was undertaken to examine whether short-term (24-h) lithium pretreatment is neuroprotective in the QA-excitotoxicity model of HD, and then to characterize the specific striatal cell populations protected by lithium and to study the possible underlying mechanisms. In addition, we investigated whether the beneficial effects of lithium in the QA model of HD involve its ability to stimulate neurogenesis and/or cell migration.

Experimental procedures

Lithium treatment and intrastriatal QA injection

Sprague–Dawley male rats (240–260 g) were obtained from Taconic Farms (Germantown, NY, USA). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce statistically meaningful results. In all experiments, lithium chloride (LiCl) dissolved in normal saline was injected at doses of 0.5, 1.0, 2.0 or 3.0 mEq/kg subcutaneously 24 h prior to and then 1 h after intrastriatal injection of QA. Normal saline injections were used as the controls. Serum lithium concentrations were determined by atomic absorption from blood samples collected at 12 and 24 h after the lithium injection (American Medical Laboratories, Chantilly, VA, USA).

Stereotaxic administration of QA was performed as previously described.[7,22] Briefly, rats were anesthetized with nembutal (50 mg/kg, i.p.), and 1 μl of normal saline containing 30 nmol of QA was infused into the left striatum in accordance with the following coordinates: 1.0 mm anterior from the bregma, 2.0 mm from the midline and 5.2 mm ventral to the dura. Sham-injection animals were infused with 1 μl of normal saline. At 7 days after infusion, rats were euthanized by decapitation or lethal injection of nembutal (100 mg/kg, i.p.).

Assessment of DNA fragmentation by electrophoresis

Electrophoretic DNA fragmentation analysis was performed as described elsewhere.[7] Briefly, striatal tissues were homogenized in a buffer containing 100 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl, pH 8.0, 0.5% SDS, and treated with 0.5 μg/ml RNase. Homogenates were incubated at 55°C, and then DNA was extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and precipitated with isopropanol and ammonium acetate. DNA precipitates were collected by centrifugation and the pellets were washed with precooled 80% alcohol, vacuum-dried, and resuspended in TE buffer. Extracted DNA was loaded (10 μg per lane) on 2% agarose gel (3 : 1; NuSieve, Rockland, ME, USA) and electrophoresed for 3 h. Separated DNA fragments were stained with ethidium bromide and visualized under UV light. DNA samples from two animals were pooled for each experimental point.

Nissl and NADPH-diaphorase (NADPH-d) staining

Modified NADPH-d histochemistry was performed as described by Ikeda *et al*.[26] Briefly, deeply anesthetized animals were perfused with ice-cold 0.1 M phosphate buffer (PB), pH 7.4, followed by 4% paraformaldehyde, 0.125% glutaraldehyde and 2% glycerol in the cold PB. The brains were postfixed by immersion in the same fixative for 1.5 h and placed overnight in 25% glycerol in phosphate-buffered saline (PBS) at 4°C. Coronal sections of 30 μm were cut with a cryostat and washed with PBS followed by incubation for 60 min at 37°C in PBS containing 1 mM β-NADPH, 0.1 mM nitro blue tetrazolium and 0.8% Triton X-100. Adjacent sections were stained with 0.1% cresyl violet using standard procedure for Nissl staining. For quantification of NADPH-d labeled cells, overlapping images covering the whole striatum area of each section were captured using a digital camera (RT Slider Spot, Diagnostic Instruments, Sterling Heights, MI, USA). All NADPH-d-positive cells in both the QA-injected and contralateral (intact) striatum were tagged and counted using Image Tool software, Version 2.0. (University of Texas Science Center in San Antonio, San Antonio, TX, USA). In each section, the number of NADPH-d-expressing cells in the injured-striata was normalized to the number of NADPH-d-positive cells in the contralateral striatum. To obtain the average number of labeled cells per striatal area, four sections from each brain were analyzed. The mean value for each group of animals was then calculated. Statistical significance was assessed by *t*-test.

Receptor autoradiography

The D_1 dopamine receptor radioligand binding assay was performed as described elsewhere[22] to measure the size of QA-induced lesions. Briefly, after decapitation, brains were extracted and flash frozen in dry ice, and 20-μm serial coronal sections were cut with a cryostat, mounted on silanated glass slides and labeled with [^{3}H]SCH-23390 (Amersham Pharmacia Biotech, Denver, CO, USA). Nonspecific binding was determined in the presence of 2 μM unlabeled SCH-23390 (RBI, Natick, MA, USA) and subtracted from total binding. For data analysis, six coronal brain sections containing the striatum were made at bregma coordinates 2.2, 1.7, 1.2, 0.7, 0.2 and −0.3 mm. Digital images of autoradiograms were captured and analyzed using a CCD camera (Sierra Scientific, Sunnyvale, CA, USA) and NIH Image software (Version 1.62). The lesion size was normalized to the contralateral (intact) striatal area and expressed as mean $\pm$ SEM of percent of control (lesioned striatal area/total contralateral striatal area $\times$ 100%). Statistical significance was assessed by ANOVA followed by Student–Newman–Keul multiple comparison test.

TUNEL and caspase-3 labeling

For terminal deoxynucleotide transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL), 10-μm coronal cryosections mounted on glass slides were

fixed in 1% paraformaldehyde in PBS, and after washing in PBS, postfixed in a mixture of ethanol and acetic acid (2 : 1) at −20°C. TUNEL assay was performed by using a Fluorescein ApopTag kit (Intergen, Purchase, NY, USA) according to the manufacture's instructions, using affinity-purified fluorescein-conjugated anti-digoxegenin sheep polyclonal antibody with the Fc portion removed. For fluorescent counterstaining, TUNEL sections were mounted in Vectashield medium containing 1.5 μg/ml propidium iodide (PI, Vector Laboratories, Burlingame, CA, USA). Selected TUNEL sections were double immunostained for the neuronal marker protein *neu*ronal *n*uclei (NeuN) using a monoclonal mouse antibody (1 : 500; Chemicon International, Temecula, CA, USA) at 4°C overnight, and Alexa Fluor 546 conjugated F(ab′)$_2$ fragment of goat anti-mouse IgG (1 : 200; Molecular Probe, Eugene, OR, USA) for 1 h at room temperature.

Adjacent sections from the same animals were stained with polyclonal rabbit antibody against the activated (cleaved) form of caspase-3 (1 : 100; Cell Signalling, Beverly, MA, USA). After blocking in 5% normal goat serum (NGS) in PBS with 0.5% Triton X-100 for 1 h at room temperature, the antibody was applied for 48 h at 4°C. Double immunostaining for NeuN was performed as described above. For signal visualization, sections were washed in PBS and incubated for 2 h at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit F(ab′)$_2$ fragment and/or Alexa Fluor 546-conjugated goat anti-mouse IgG F(ab′)$_2$ fragment (Molecular Probe). Both antibodies were diluted at 1 : 200 and dissolved in PBS containing 0.5% Triton X-100 and 1% NGS.

For quantitative analysis, images of lesioned striatum at the level of the QA-injection site were captured using a digital camera (RT Slider Spot, Diagnostic Instruments, Sterling Heights, MI, USA). Quantification was performed with Image Tool software, Version 2.0. (University of Texas Science Center in San Antonio, San Antonio, TX, USA). The total number of immunolabeled cells per 0.52 mm^2 for TUNEL/NeuN labeling and 1.05 mm^2 for TUNEL/PI and caspase-3 labeling were calculated. To obtain the average number of labeled nuclei, four sections at the level of the injection from each brain were analyzed. The number of cells in each section was corrected using the Abercrombie equation.[27] The mean value for each group of animals was then calculated. Statistical significance was assessed by ANOVA with Bonferroni *post hoc* test or a two-tailed Student's *t*-test.

Bcl-2 and Bax immunohistochemistry

For Bcl-2 and Bax immunohistochemistry, animals were overanesthetized and perfused with 4% paraformaldehyde in 0.1 M PB. Brains were removed and 30-μm sections were cut with a cryostat. Free-floating sections were incubated with a rabbit polyclonal antibody against Bcl-2 or Bax in PBS at 4°C for 48 h at a dilution of 1 : 100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1% NGS and 0.3% Triton X-100. The immunoreactive products were visualized using a biotinylated goat anti-rabbit antibody and avidin-conjugated horseradish peroxidase complex (Vectastain Elite Kit, Vector Laboratories), according to the manufacture's protocol.

Double immufluorescent staining of cells for Bax and anti-microtubule-associated protein 2 (MAP2) was performed by incubating sections with a mixture of polyclonal anti-Bax rabbit antibody (1 : 100) and mouse anti-MAP2 monoclonal antibody (1 : 200 dilution; Chemicon) at 4°C overnight. Signal visualization was performed as described in the previous section using the proper combination of anti-mouse and anti-rabbit secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 546 (1 : 200 dilution; Molecular Probe).

For quantitative off-line analysis of Bcl-2 staining, 14–18 overlapping images covering the whole striatum area of a section were captured using a digital camera and analyzed using the NIH Image software (Version 1.62). For all Bcl-2-labeled striatal neurons, mean pixel intensity values were collected within the soma whose outlines had been previously traced under bright-field illumination. In each section, three randomly chosen areas of the striatum, free of labeled neurons, were averaged for background subtraction. The final value was derived by averaging two measurements in each cell profile. Histogram distribution analysis and statistical tests were performed with Origin 4.1 (Microcal) software.

Western blotting

Western blotting was performed as described previously.[22] Briefly, the striata from both hemispheres of each individual brain were separately dissected, homogenized and sonicated in lysing buffer. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were mixed with loading buffer and boiled for 5 min before separation on a 4–20% Tris-glycine gel by electrophoresis. After proteins were transferred to a PVDF membrane, the blots were incubated with a rabbit polyclonal antibody against Bcl-2 or a mouse monoclonal antibody against Bax at 1 : 1000 dilution (Santa Cruz Biotechnology), and then probed with a horse anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (Vector Laboratories). Detection was made using an ECL kit (Amersham Pharmacia Biotech, Piscatway, NJ, USA). Quantification of Western blots was performed using a Kodak Image Station 440CF and KDS1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA).

Bromodeoxyuridinie (BrdU) labeling

BrdU was administered intraperitoneally (50 mg/kg, Sigma Chemical, St Louis, MO, USA), with the first injection given immediately after QA infusion, a second injection given 2 h later, and followed by daily injections over the next 4 days. At 3 days after the last BrdU injection (7 days after QA treatment),

the animals were overanesthetized and perfused with 4% paraformaldehyde in 0.1 M PBS. Brains were then postfixed by immersion into the same fixative containing 10% sucrose overnight and then cut into 30-μm sections with a cryostat. For immunochemical visualization of BrdU, sections were pretreated in 2N HCl at 37°C for 40 min, blocked with 10% NGS at room temperature for 1 h, and then incubated with mouse anti-BrdU antibody (1 : 100, BD Biosciences Pharmingen, San Diego, CA, USA) in PBS with 0.5% Triton X-100 and 1% NGS for 48 h. For double immunostaining, sections were treated at 4°C overnight with a combination of monoclonal mouse anti-BrdU and polyclonal rabbit anti-glial fibrillary acidic protein (anti-GFAP) antibodies at 1 : 1000 dilution, or monoclonal rat anti-BrdU at 1 : 100 dilution (Accurate Chemical, Westbury, NY, USA) and monoclonal mouse anti-NeuN antibodies at 1 : 1000 dilution in PBS with 0.5% Triton X-100 and 1% NGS. Signal visualization was performed as described in the previous sections using the appropriate combination of anti-rat, anti-mouse or anti-rabbit secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 546 (1 : 200 dilution; Molecular Probe).

Results

Damaging effects of QA on striatal DNA

DNA laddering, a hallmark of apoptosis, was used as an index for selecting the optimal dose of QA and time after injection to assess striatal degeneration and lithium neuroprotection. Within the first 3 days after infusion with 60 or 120 nmol of QA, a considerable increase was observed in the levels of internucleosomal DNA cleavage (Figure 1). However, the striatal DNA was randomly and completely degraded 7 days after QA infusion at these doses, as shown by smear effect on gel electrophoresis, suggesting the occurrence of massive necrotic cell death. Injection of 30 nmol QA, on the other hand, induced a low level of DNA laddering at 7 days, but not at 3 days after infusion. This level of apoptotic cell death correlated with the results of Nissl staining, NeuN immunohistochemistry and caspase-3 activation assays (see below) that showed selective neuronal degeneration in the striatum. Thus, a paradigm of 7 days after infusion with 30 nmol QA was chosen as the end point in our studies on lithium neuroprotection in the QA-injection model of HD.

Plasma concentration of lithium

To assess the blood levels of lithium, we measured the plasma concentration of lithium in a group of six rats at 12 and 24 h after subcutaneous injections of three different doses of this drug. At 12 h after treatment with 1.0, 2.0 or 3.0 mEq/kg of LiCl, its plasma levels were 0.47 ± 0.06, 0.60 ± 0.04 or 1.04 ± 0.04 mM, respectively. Plasma levels of lithium measured 24 h after injection of 1.0, 2.0 or 3.0 mEq/kg were 0.19 ± 0.01, 0.33 ± 0.01 and 0.79 ± 0.01 mM, respectively. Thus, at these two time points, the plasma concentrations of lithium at these doses were either within or less than therapeutic plasma levels, namely, 0.4–1.2 mM.

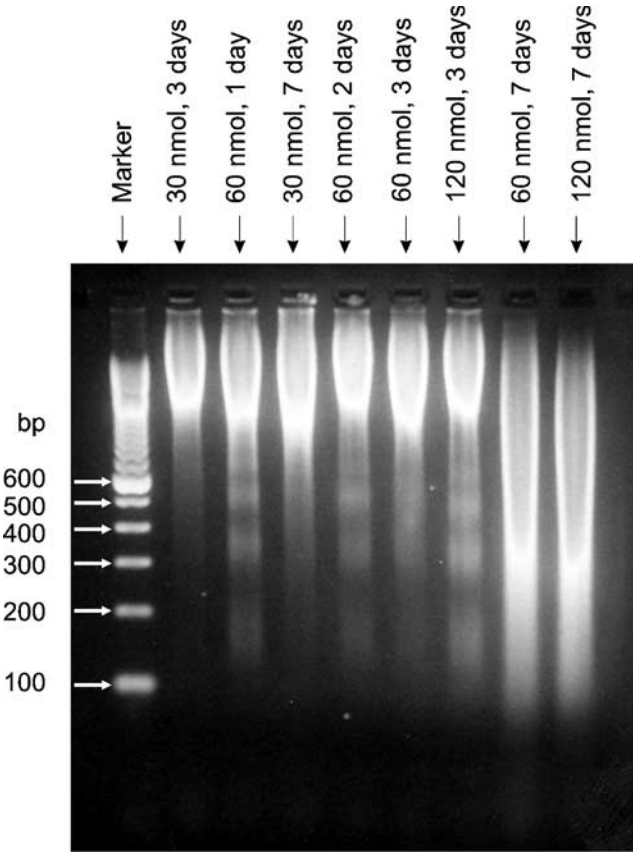

Figure 1 Damaging effect of QA on striatal DNA. DNA laddering, a hallmark of apoptosis, was used as an index for selecting both the dose for QA infusion and the optimal time after QA injection to assess striatal degeneration. DNA fragments (10 μg per lane) were separated by electrophoresis a on 2% agarose gel. DNA samples from two animals were pooled for each experimental point. The lane to the far left was loaded with DNA standards. Lanes to the right were loaded with DNA extracted from QA-injected striatal tissue. Animals were euthanized 1, 2, 3 or 7 days after the intrastriatal injection of 30, 60 or 120 nmol as indicated above each lane. Note that, while a considerable increase in internucleosomal DNA cleavage was observed within the first 3 days after infusion with 60 or 120 nmol of QA, the DNA cleavage became random and extensive 7 days after QA infusion. On the other hand, 30 nmol of QA induced a DNA laddering at 7 days, but not at 3 days after infusion.

Effects of lithium on QA-induced striatal lesion

An examination of Nissl-stained sections produced a clear demonstration of QA-induced neuronal degeneration in the striatum (Figure 2b) as compared with the undamaged contralateral striatum (Figure 2a). Subcutaneous injections of 3.0 mEq/kg of LiCl 24 h prior to and 1 h after QA infusion resulted in a partial prevention of neuronal loss in the injured area (Figure 2c). Lithium pretreatment also reduced by up to 40%

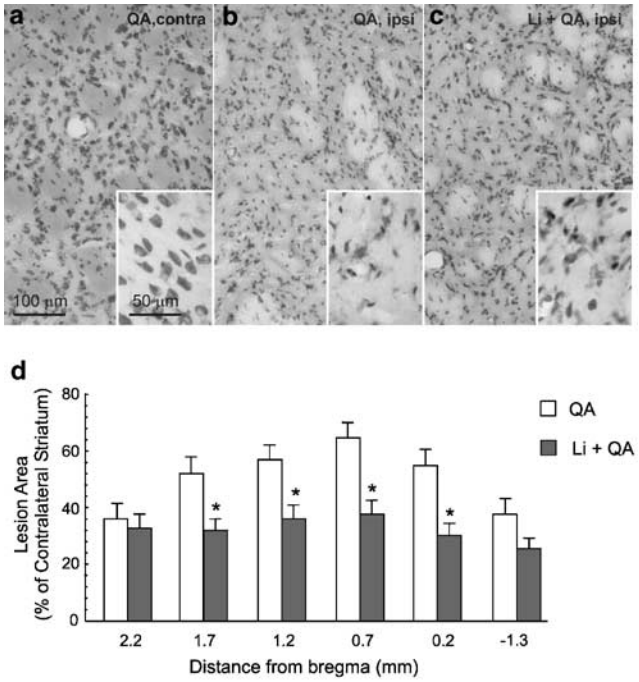

Figure 2 Neuroprotective effect of lithium in QA-injected striata. (a–c), Representative Nissl staining of the striata of rats injected with QA (30 nmol) and examined 7 days later. (a) and (b) show contralateral (intact) and ipsilateral (injured) striatal sections, respectively, from rats without lithium treatment; (c) shows the ipsilateral (injured) striatum from a rat pretreated with 3.0 mEq/kg of lithium on the day prior to QA infusion and 1 h thereafter. Three or more animals were used in each group. Inset in each panel shows high magnification image of striatal cells. (d) Measurements of the size of QA-induced striatal lesion in animals with or without 3.0 mEq/kg LiCl pretreatment, as determined by D_1 dopamine receptor autoradiography. Lesion sizes, defined as described in the Experimental procedures, were normalized to the contralateral striatum and expressed as mean $\pm$ SEM of percent of contralateral control. Results were derived using eight rats in the control and 12 rats in the lithium-pretreated group. Note that lithium pretreatment decreased the size of QA-induced lesions. *, $P<0.05$; as compared with the corresponding saline control.

the size of the area affected by QA infusion as assessed by D_1 dopamine receptor binding assay in sequential striatal sections (Figure 2d). Previous studies have established that striatal medium-sized spiny neurons express D_1 dopamine receptors and that D_1 receptor autoradiography is a valid method for quantifying QA-induced striatal lesions.[7,22,28]

Lack of effect of lithium pretreatment on NADPH-d-expressing interneurons

We employed NADPH-d histochemistry to investigate whether lithium protection is limited to striatal medium spiny projection neurons (Figure 3). We found that QA injection induced more than 30% cell loss of striatal interneurons expressing NADPH-d as compared with the undamaged contralateral striatum. The surviving NADPH-d-positive interneurons in the lesioned area of the striatum (Figure 3b and b') appeared to have shrunken cell bodies and shorter processes as compared with the neurons in the contralateral (intact) striatum (Figure 3a and a'). While the treatment with 3 mM mEq/kg of LiCl seemed to improve NADPH-d cell morphology in the injured striatum (Figure 3d and d'), the neurons still had smaller somatic area and less-developed dendritic tree as compared with the contralateral striatum (Figure 3c and c'). Quantitative analysis of NADPH-d-positive cells showed that lithium treatment did not significantly reduce the loss of NADPH-d-positive neurons induced by QA injection (Figure 3E).

Lithium effects on neuronal DNA damage and cell death in QA-infused striatum

We next investigated the effects of different doses of LiCl (0.5, 1.0, 2.0 and 3.0 mEq/kg) on DNA damage and neuronal cell death in QA-infused rats. While there was no TUNEL cells in the contralateral (intact) striatum (Figure 4a), QA injection produced a significant number of cells with DNA damage (Figure 4b). Lithium at 1 mEq/kg was sufficient to reduce the number of striatal TUNEL cells by more than 40% (Figure 4c and d), while higher doses of lithium failed to produce further reductions in DNA damage (Figure 4d). To assess whether the decrease in QA-induced TUNEL was specifically associated with an increase in neuronal survival, we also stained the TUNEL sections for NeuN,[29] a neuron-specific nuclear protein. NeuN was specifically chosen for this study because this protein is present in most neuronal cell subtypes throughout the central nervous system, including striatum (Figure 4a), and is preferentially expressed in nuclei, making it an excellent marker for quantitative neuronal analysis. The results showed that lithium pretreatment significantly increased the number of NeuN-labeled cells by approximately 80% at these four doses examined (0.5–3.0 mEq/kg; Figure 4c and e) as compared with the saline control (Figure 4b and e). An inverse correlation between TUNEL and NeuN labeling in the striatum suggested that TUNEL cell were neurons (Figure 4f). Indeed, confocal microscopy analysis showed that the majority ($>80\%$) of TUNEL-positive cells were double labeled for NeuN (Figure 5a and b). Confocal scanning under higher magnification revealed that many of the TUNEL-positive nuclei near the QA-injection site were found in disintegrated cells (Figure 5c and c') and this effect was largely prevented by lithium (Figure 5d and d'). To examine possible changes in striatal cell density after lithium treatment, we counterstained TUNEL sections with PI in QA-infused striatum (Figure 6a and b). Quantitative analysis showed that, while lithium significantly decreased the absolute number of TUNEL nuclei per area, the total numbers of nuclei in the striata of saline- and lithium-pretreated animals were not significantly different (Figure 6c).

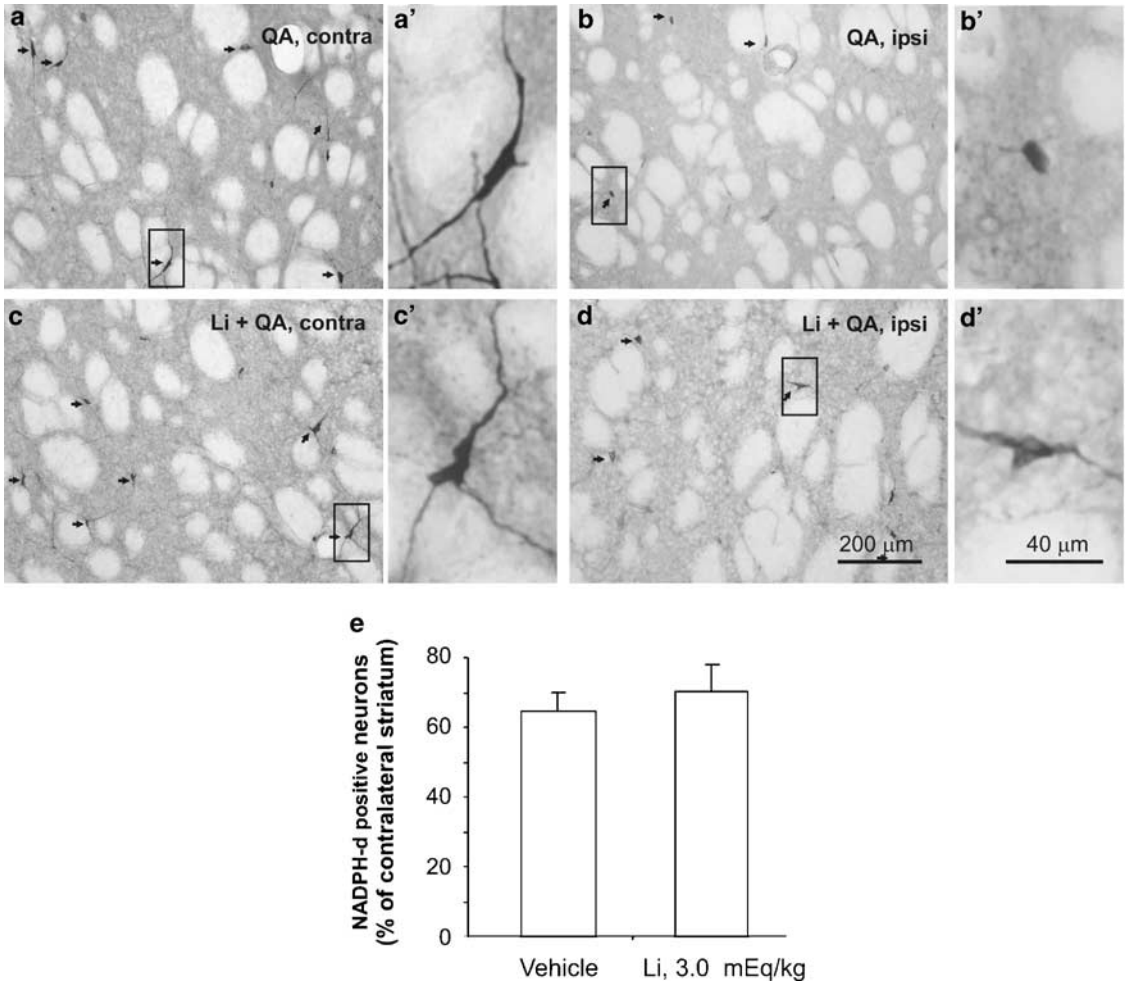

Figure 3 Lithium pretreatment does not protect NADPH-d- expressing neurons in QA-injected striatum. (a–d'), Representative images of NADPH-d-positive neurons in contra- and ipsilateral striata of rats pretreated with saline (a–b') and LiCl (3.0 mEq/kg; c–d'). Boxed regions in (a), (b), (c) and (d) are shown as enlarged images in (a'), (b'), (c') and (d'), respectively. (e) Results of a quantitative analysis of the loss of NADPH-d-expressing neurons. To obtain the average number of labeled cells per each striatum, four sections from each brain were analyzed. In each section, the number of NADPH-d-expressing cells in both the QA-injected and contralateral (intact) striatum were counted. The numbers of NADPH-d-expressing cells in the injured striata were normalized to the contralateral (intact) striatum. Three animals were used in each group. Shown are means in each group.

Lithium effects on caspase-3 activity in the injured striatum

Caspases are present in living cells as an inactive proenzyme form. Once processed by proteolytic cleavage, they are activated and irreversibly committed to the cleavage of various proteins key to the functioning of the cell.[30] Caspase-3, a downstream cleavage product of the caspase cascade, plays a central role in the execution of apoptotic cell death. We assessed the effects of lithium on the activation of caspase-3 in QA-infused striata derived from the five groups of animals described in the previous section. These results showed extensive immunolabeling of activated caspase-3 in QA-infused striatal sections (Figure 7a) and a marked reduction in the intensity of labeling in the corresponding sections derived from rats pretreated with different doses of lithium (Figure 7b and c). Quantification of these data (Figure 7d) show the dose dependence of the ability of lithium to reduce caspase-3 activation: 1, 2 and 3 mEq/kg of lithium resulted in 40, 50 and 56% reduction in the number of cells positive for activated caspase-3, respectively. Double staining of these striatal sections with antibodies specific for activated caspase-3 and NeuN revealed that caspase-3 was activated exclusively in neurons (Figure 7e). Sham-operated rats did not show caspase-3 activation in their striatal sections (data not shown).

Lithium effects on Bcl-2 and Bax expression in injured striatum

In an attempt to explore potential neuroprotective mechanisms of lithium, we examined the effects of QA and lithium on the expression of antiapoptotic Bcl-2 and proapoptotic Bax using Western blotting and immunohistochemistry. Western blot analysis of

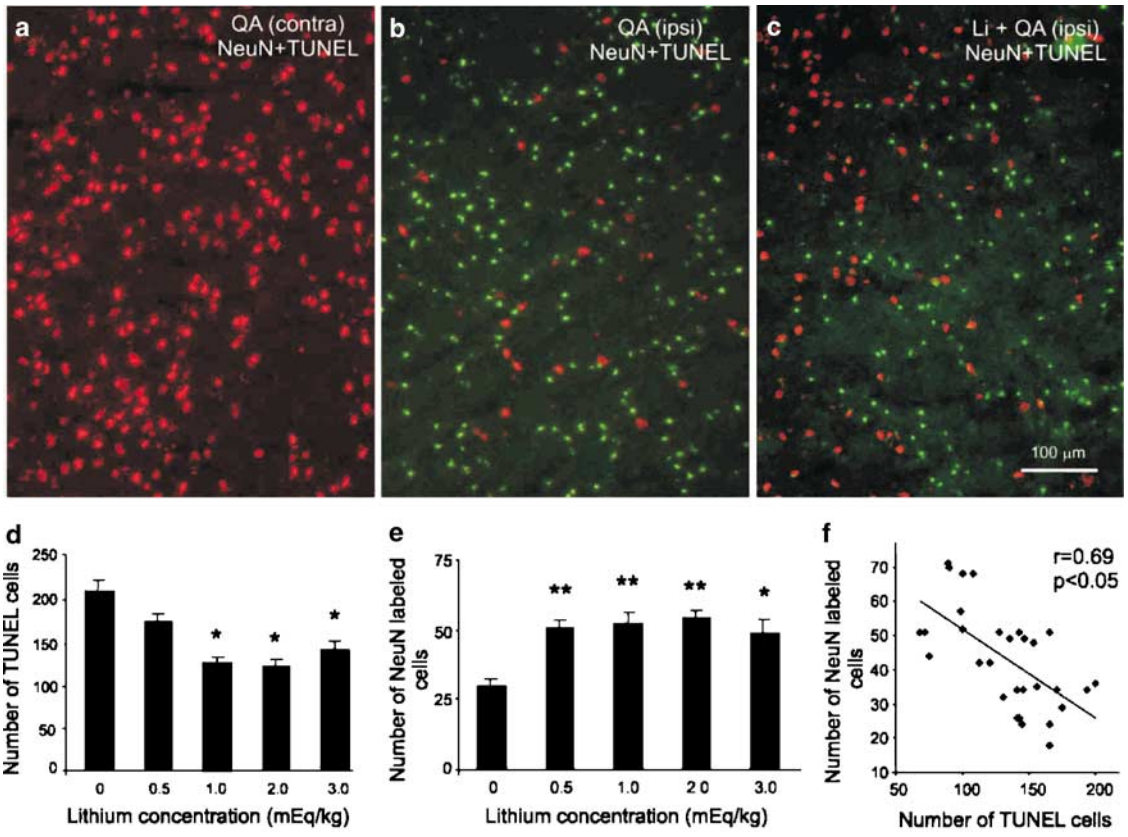

Figure 4 Lithium pretreatment protects against neuronal DNA damage in QA-injected striatum. (a–c), Representative fluorescent images of striata in the rats injected with QA. (a) and (b) show images of sections from contralateral (intact), and ipsilateral (injured) striata, respectively, of rats without lithium pretreatment. (c) shows an image from an ipsilateral (injured) striatum of an animal pretreated with 1 mEq/kg of lithium. Green and red fluorescence indicates TUNEL and NeuN labeling, respectively. (d) Numbers of TUNEL cells in striata of animals pretreated with the indicated doses of LiCl. (e) Numbers of NeuN-labeled cells in the same sections as in (d). Three or more animals were used in each group. *, $P<0.05$; **, $P<0.01$, compared with the saline control. (f) Correlation analysis of TUNEL and NeuN labeling in QA-injected striata. Each point represents data from one section. In total, 30sections (two section per animal) were pooled from five groups of animals: one was treated with saline and four were treated with the doses of lithium as indicated in (d) and (e). Significant inverse correlation ($r=0.69$, $P<0.05$) suggests that neuronal depletion in QA-injured striata is associated with DNA damage.

striatal Bcl-2 protein levels in rats infused with QA and pretreated with 3.0 mEq/kg showed a significant increase in total Bcl-2 content as compared with either saline-treated or sham-operated rats (Figure 8a). In contrast, QA infusion caused a slight but significant decrease in striatal Bcl-2 protein levels. We also investigated the effects of different doses of LiCl (0.5, 1.0, 2.0 and 3.0 mEq/kg) on increases in Bcl-2 protein levels and found that the increase was significant at 0.5 mEq/kg and maximal at 1–2 mEq/kg as compared with saline-treated animals (Figure 8b).

Next, we immunostained the brain sections of QA-injected rats for Bcl-2. Based on visual inspection of stained cells, lithium induced increases in Bcl-2-immunostaining in neurons of striata both ipsilateral and contralateral to the site of QA injection, (Figure 9a–c). Therefore, we performed detailed quantitative analysis of Bcl-2 immunoreactivity within the soma of QA-injured neurons in the ipsilateral striata based on density of immunostaining. We found about a 40% increase in the number of Bcl-2-expressing striatal neurons (from 122 to 173; Figure 9d), and a 60% augmentation in signal intensity within each neuronal soma (Figure 9f) in QA-injured striata in animals pretreated with lithium as compared with a saline-pretreated control. In contrast, lithium pretreatment induced a 24% expansion in the population of Bcl-2-expressing neurons (from 209 to 260; Figure 9e) without affecting the Bcl-2 content per neuron (Figure 9f) in the contralateral striatum as compared with saline-pretreated control.

Western blot analysis of striatal Bax protein levels in QA-injected rats with or without lithium pretreatment revealed that lithium pretreatment did not significantly change Bax contents in injured striata (Figure 10a). To clarify these data, we immunostained for Bax using brain sections derived from QA-infused animals. Immunohistochemical results showed the presence of a relatively small number of Bax-positive cells with neuron-like morphology in the contralateral striata of both lithium (not shown)- and saline (Figure 10b and b')-pretreated animals. In QA-infused

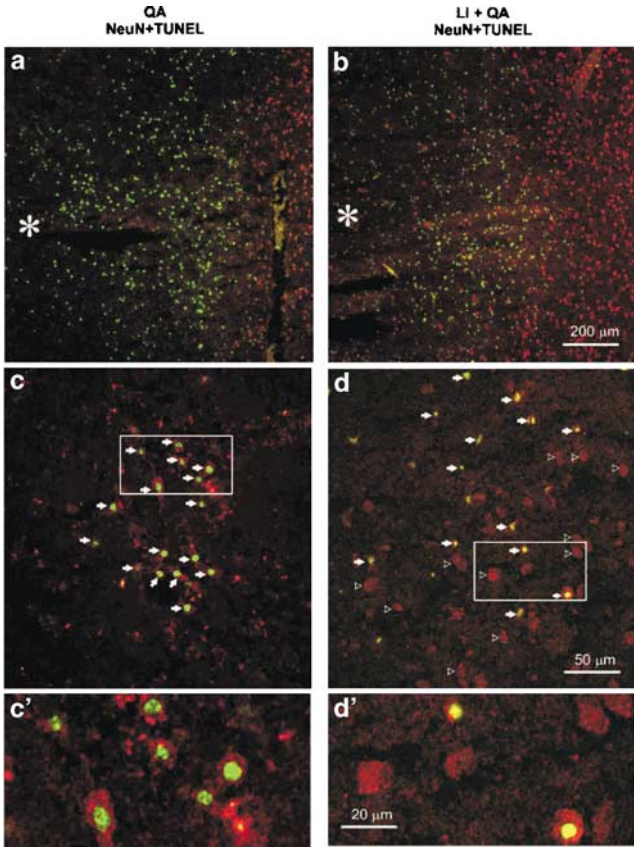

Figure 5 Comparative confocal microscopy analysis of double TUNEL and NeuN labeling reveals that lithium decreases QA-induced neuronal DNA damage in the striatum. (a,b) Low magnification images revealing a large population of TUNEL-staining (green) cells also staining for NeuN (red) near the site of QA injection (indicated by asterisks). (c,d) Confocal images of higher magnification showing that the majority (>80%) of TUNEL-stained cells were also labeled for NeuN. Arrows (c,d) indicate TUNEL-positive neurons and arrowheads (d) indicate neurons positive for NeuN, but negative for TUNEL. Boxed regions in (c,d) are shown as enlarged images in c' and d', respectively. In saline-treated striatum (c'), note the absence of neuronal profiles without TUNEL and the disintegrated neuronal profiles where TUNEL and NeuN are in close apposition, as compared with lithium-treated saline (d').

striatum of saline-treated animals, we observed few or no Bax-positive cells with neuron-like morphology, but detected immunostaining in smaller cells with glia-like morphology (Figure 10c and c'). Interestingly, in QA-injected striatum of lithium-treated animals, we observed Bax-positive cells with neuron-like morphology (Figure 10d and d') similar to those observed in contralateral striatum. Antibodies against Bax and the neuron-specific microtubule-associated protein MAP2 confirmed that the labeled cells were neurons (Figure 10e). The relatively low levels of staining did not allow reliable quantitative comparisons between the intensities of neuronal staining in the QA-infused and contralateral striata of lithium-treated animals.

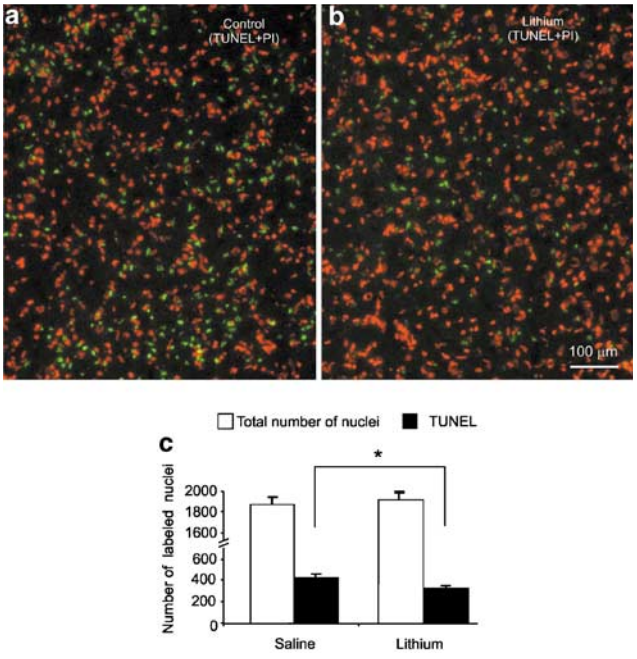

Figure 6 Lithium does not change nuclei density in QA-infused striatum. (a,b) Representative fluorescent images of TUNEL-stained sections counterstained with PI from QA-infused striata of rats pretreated with saline and lithium, respectively. Red fluorescence, PI labeling; Green or yellow fluorescence, TUNEL. (c) Results of quantitative analysis are plotted as the numbers of TUNEL- and PI-labeled cells in the rats treated with saline and LiCl (3 mEq/kg) ($n=3$ in each group). *, $P<0.05$, compared with the saline group. Note that lithium treatment decreased the number of TUNEL cells compared to saline-treated controls. There was no difference, however, in the total number of nuclei measured by the sum of those labeled by PI and TUNEL in the two groups of animals.

Effects of lithium on cell proliferation

In light of the ability of lithium to stimulate neurogenesis *in vivo*[23] and to enhance neuronal progenitor proliferation *in vitro*,[24,25] the effects of lithium on neurogenesis in the QA-injured striatum were examined. We injected rats with the proliferation marker BrdU complemented by staining the sections for the neuronal marker NeuN or the astroglial marker GFAP, to identify dividing progenitor cells. At 1 week after QA infusion, BrdU-positive cells were rare in the injured striatum near the QA injection site in rats not treated with lithium (Figure 11a). In the same sections, however, densely packed BrdU-labeled cells were observed in the subventricular zone (SVZ) of both ipsilateral (QA-injected, Figure 11b) and contralateral (intact, Figure 11c) striata. These BrdU-labeled cells were likely undifferentiated progenitor cells because they were not recognized by either GFAP- or NeuN-specific antibodies. On the other hand, we found a large number of BrdU-labeled cells in the QA-injected striata of rats pretreated with 3 mEq/kg of lithium (Figure 11d). This increase in BrdU labeling near the QA injection site of damaged

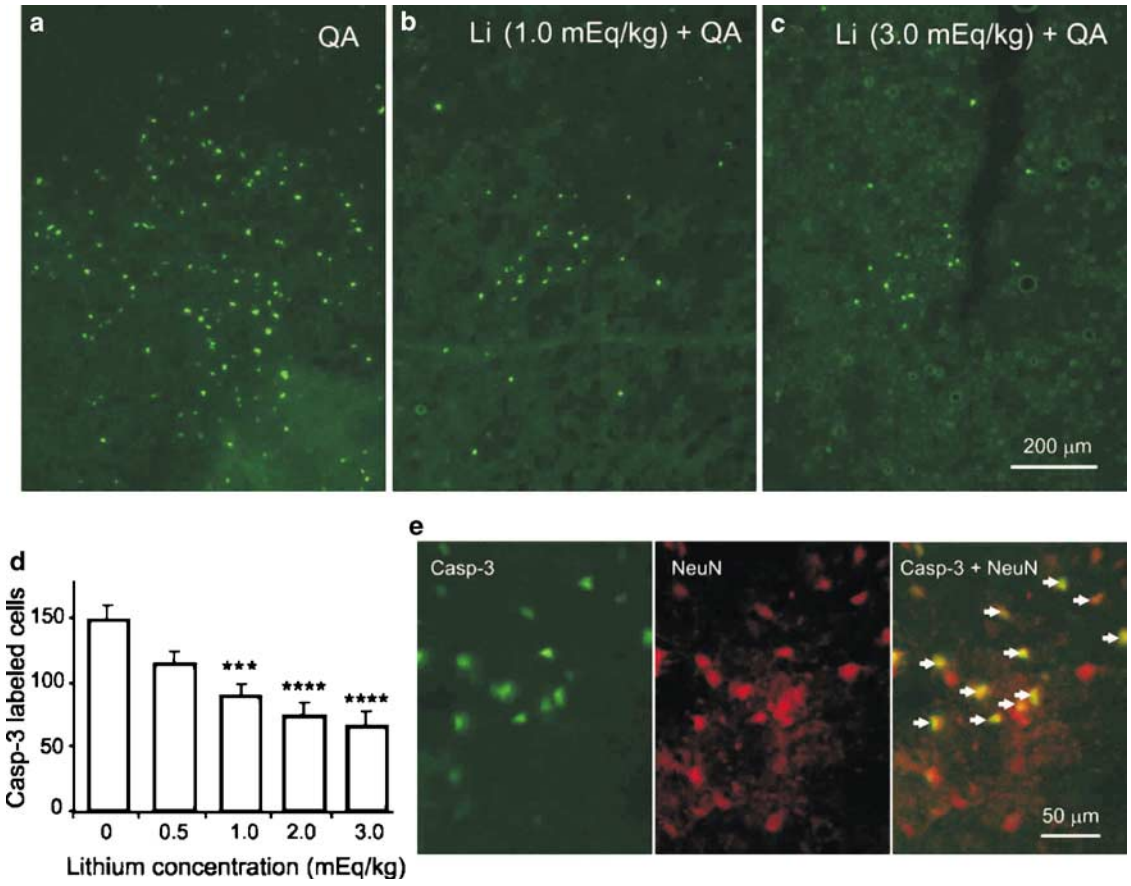

Figure 7 Lithium pretreatment dose-dependently decreases caspase-3 activation in QA-infused rat striatum. A-C, Representative sections showing immunostaining of cells containing activated caspase-3 in animals without lithium pretreatment (a), treated with 1.0 mEq/kg (b) or 3.0 mEq/kg (c) of lithium. (d) The quantification of cells labeled for activated caspase-3 in the QA-infused striata of animals treated with different doses of lithium. Three or more animals were used in each group. ***, $P<0.005$; ****, $P<0.001$, compared with saline control. E. Double immunostaining with antibodies against caspase-3 and NeuN reveals that caspase-3 was activated almost exclusively in neurons. Arrows indicate double-labeled cells.

striata was associated with a reduction in the numbers of BrdU-labeled cells in the SVZ (Figure 11e). In the contralateral (intact) striatum of lithium-pretreated rats, a smaller degree of decrease in the number of BrdU-labeled cells within the SVZ and an increase in BrdU labeling just outside the SVZ were also observed (Figure 11f). The brightness and size of the BrdU labeling were similar to those found in intestinal epithelium used as the positive control (Figure 11g). Next, we used double labelling for BrdU and GFAP or BrdU and NeuN, respectively, to screen striatal cells for new-born astocytes and neurons. Double immunostaining for BrdU and GFAP demonstrated that most proliferating cells were not astrocytes (Figure 12a–c). Some GFAP-expressing cells, however, were weakly labeled with BrdU (Figure 12c). These double-labeled cells were located primarily in the vicinity of the QA-injection site. While the great majority of the BrdU-labeled (proliferating) cells were not mature neurons, we consistently observed the presence of a small population of NeuN-stained cells with strong BrdU labeling (Figure 12d–f). In contrast to the GFAP-labeled cells, these cells were located slightly to the periphery of the damaged area.

Discussion

A prominent feature of QA-induced excitotoxicity in the striatum is the occurrence of apoptosis characterized by the internucleosomal cleavage of DNA.[7] Here, we found that injections of 30 nmol QA resulted in a low degree of DNA laddering at the end of 7 days, and produced significant cell loss shown by Nissl staining of striatal sections. Furthermore, the neuroprotective effect of lithium could be clearly observed at this dose of QA. Although lithium's neuroprotective effects against excitotoxicity in cultured neurons require long-term pretreatment, experiments using a rat ischemia model of middle cerebral artery occlusion demonstrated that the neuroprotective effect can be achieved by administrating the drug immediately after the ischemic insult.[19] For this reason, we injected lithium subcutaneously in this study 24 h prior to and 1 h after QA infusion. Medium-sized

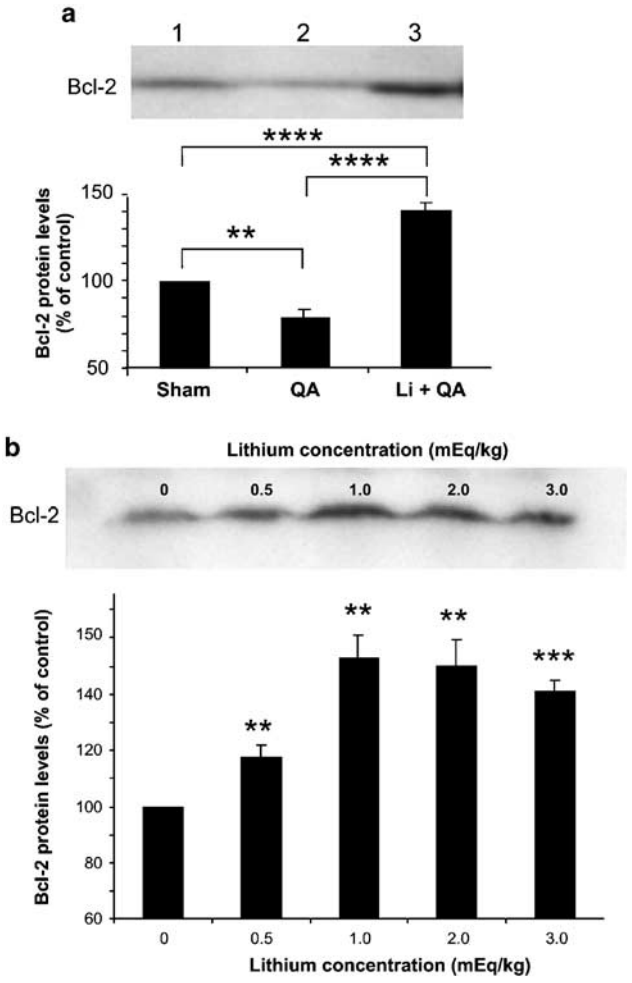

Figure 8 Western blot analysis of striatal Bcl-2 protein levels. The upper panels in (a) and (b) show representative Western blots of Bcl-2. The lower panels show the quantified results of Bcl-2 protein levels determined by Western blotting from a group of rats. (a), The expression of Bcl-2 in the striatum of sham-operated animals (lane 1, $n=3$) compared to the striata of QA-injected animals pretreated with saline (lane 2, $n=6$) or those pretreated with 3.0 mEq/kg of lithium (lane 3, $n=4$) is shown. (b), Bcl-2 expression in QA-injected striata of animals without lithium treatment or pretreated with different (0.5, 1.0, 2.0 or 3.0 mEq/kg) doses of LiCl, as indicated above each band. Three animals were used for each dose in (b). **, $P<0.01$; ***, $P<0.005$; ****, $P<0.001$.

spiny projection neurons expressing dopamine receptors are predominantly affected in the striatum of HD patients, while NADPH-d-expressing interneurons are less vulnerable.[1] Our studies using D_1-receptor autoradiography revealed that lithium considerably decreased the loss of D_1-receptor-expressing neurons. Analysis of the number of cells expressing the neuronal marker NeuN showed that subcutaneous injections of lithium resulted in an increase of up to 80% in neuronal survival in the injured striatum. Since medium-sized spiny neurons represent the most numerous striatal neuronal cell type, we conclude that the neuroprotective effect of lithium primarily involves this neuronal population.

There has been considerable controversy regarding the vulnerability of striatal aspiny interneurons to QA.[5,6,31,32,33,34] We found a 30% depletion of NADPH-d-expressing neurons near the QA-injection site in both lithium-treated and untreated rats, indicating some degree of vulnerability of these neurons to QA. The discrepancy between our data and some of the published reports might reflect the differences in QA dosage and the longer observation time in our experiments. Notably, the vulnerability of neurons to QA excitotoxicity has been postulated to be related to higher levels of the NR2B subunit protein in the NMDA receptor complex.[11] The reason that NADPH-d-expressing neurons were not protected by lithium treatment is unclear, but this could be the result of differential requirements for drug concentration and/or treatment time. Interestingly, neuroprotection of NADPH-d-expressing neurons has been reported to require a critical level of human ciliary neurotrophic factor (CNTF) in the rat QA model of HD.[35] Moreover, lower levels of caspase-9 expression and caspase-3 activation were found in NADPH-d-positive neurons than in NADPH-d-negative neurons in response to apoptotic insult in striatal cultures.[36] These observations could be relevant to lithium's inability to protect NADPH-d-expressing neurons, as one of the major lithium effects shown in this study is to decrease QA-induced caspase-3 activation.

While both apoptosis and necrosis occur in the brain as a result of excitotoxicity, striatal neuronal loss in HD and excitotoxic animal models of HD involves primarily apoptotic cell death.[7,30,37] We found that treatment with 1.0–3.0 mEq/kg of LiCl was effective in decreasing the number of striatal neurons undergoing apoptosis as determined by TUNEL staining. Excitotoxic injury, an effect mainly mediated by activation of NMDA receptors, is associated with cellular edema.[38–40] It is therefore possible that edema or an increase in the volume of the striatum could have influenced the counts of TUNEL cells in our experiments. We believe that this is not the case because morphometric analysis of serial sections using D_1-receptor autoradiography failed to indicate a significant change in the striatal volume. This possibility was further excluded by cell-density assessments using counterstaining TUNEL sections with PI to show that there was no significant change in the density of labeled nuclei in the striatum after lithium treatment.

Although TUNEL is commonly used as a marker of apoptosis *in vivo* and *in vitro*, labeling of necrotic cells is also possible in some experimental conditions.[41] Caspase activation, on the other hand, is regarded as a specific marker of apoptosis.[30] Caspase-3, is the primary 'executioner' of apoptotic cell death and its transcriptional upregulation and activation in the transgenic mouse model of HD has been demonstrated.[42] Thus, as a specific marker of apoptosis, we used immunostaining of the activated form of

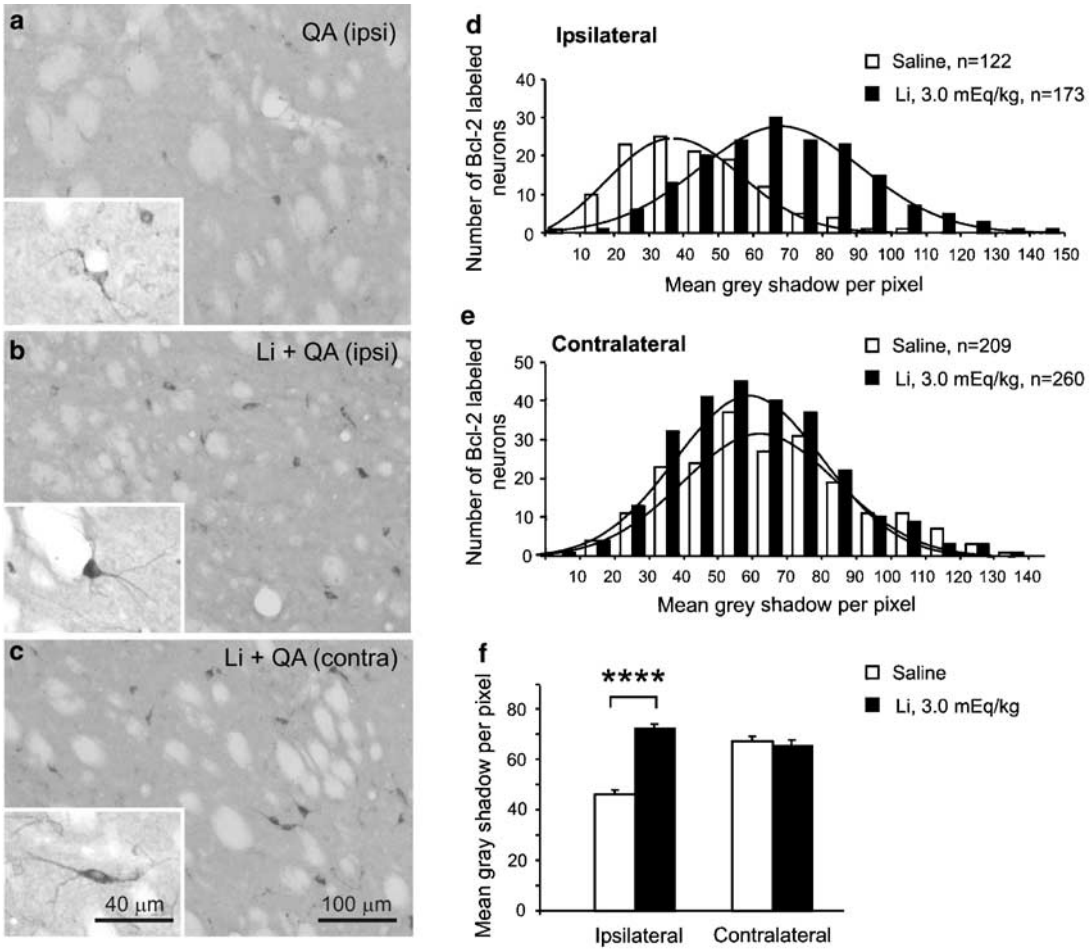

Figure 9 Image analysis of Bcl-2 immunostaining in striatum infused with QA. (a) Representative image of Bcl-2-positive cells in ipsilateral (QA-infused) striatum of saline-pretreated rat. (b,c) Representative images of Bcl-2-positive cells in ipsilateral and contralateral (intact) striata of lithium-pretreated rat. Inset in each panel shows high magnification image of representative Bcl-2-expressing striatal cell. (d,e) Distribution histograms of Bcl-expressing neurons in the ipsi- and contralateral striata of lithium and saline-pretreated rats. Note that lithium pretreatment increased the total number (*n*) of Bcl-2-labeled cells in both ipsi- and contralateral striata. In addition, lithium increased the portion of intense immunostaining Bcl-2-positive neurons in ipsilateral striata. (f) Mean intensity histograms of Bcl-2 staining in the same neuronal populations as in (d,e). Each neuronal population was pooled from 4 sections derived from two animals (two sections per animal). ****, $P<0.001$, compared with the corresponding saline control.

caspase-3. LiCl treatment at 2.0–3.0 mEq/kg was effective in decreasing caspase-3 activation in striatal neurons. The anti-apoptotic action of lithium on striatal medium spiny neurons may involve an increase in levels of Bcl-2 protein, which is known to inactivate caspase-3 through inhibition of Bax-induced cytochrome *c* release from the mitochondria.[43,44] The concentration dependence of lithium-elicited neuroprotection in rat striata closely correlated with an elevation in the Bcl-2 content of striatal tissue as assessed by Western blotting. The possibility that the increase in striatal Bcl-2 levels was due to its overexpression in non-neuronal cells or merely the result of enhanced survival of striatal neurons already expressing Bcl-2 was excluded by immunohistochemical analysis. Image analysis indicated that the lithium-induced increase in Bcl-2 immunoreactivity was found in neurons and reflected not only an increase in the number of cells expressiong Bcl-2 but also an elevation in the level of Bcl-2 expression per cell. Western blotting did not show significant effects of lithium on Bax protein levels in the injured striatum. However, we might be dealing with a complex phenomenon in which lithium has dual effects. On one hand, this drug appeared to inhibit the QA-induced death of striatal neurons including those expressing Bax. On the other hand, it might suppress the Bax immunostaining in glia-like cells. Owing to the relatively low levels of Bax immunostaining, it is difficult to perform meaningful quantitative analysis of the Bax staining in these striatal tissues.

Several antidepressant drugs[45] and lithium[23] facilitate constitutive neuronal proliferation in the rat hippocampus. Lithium also stimulates progenitor cell proliferation in cortical neuronal and cerebellar granule cell cultures.[24,25] In light of these

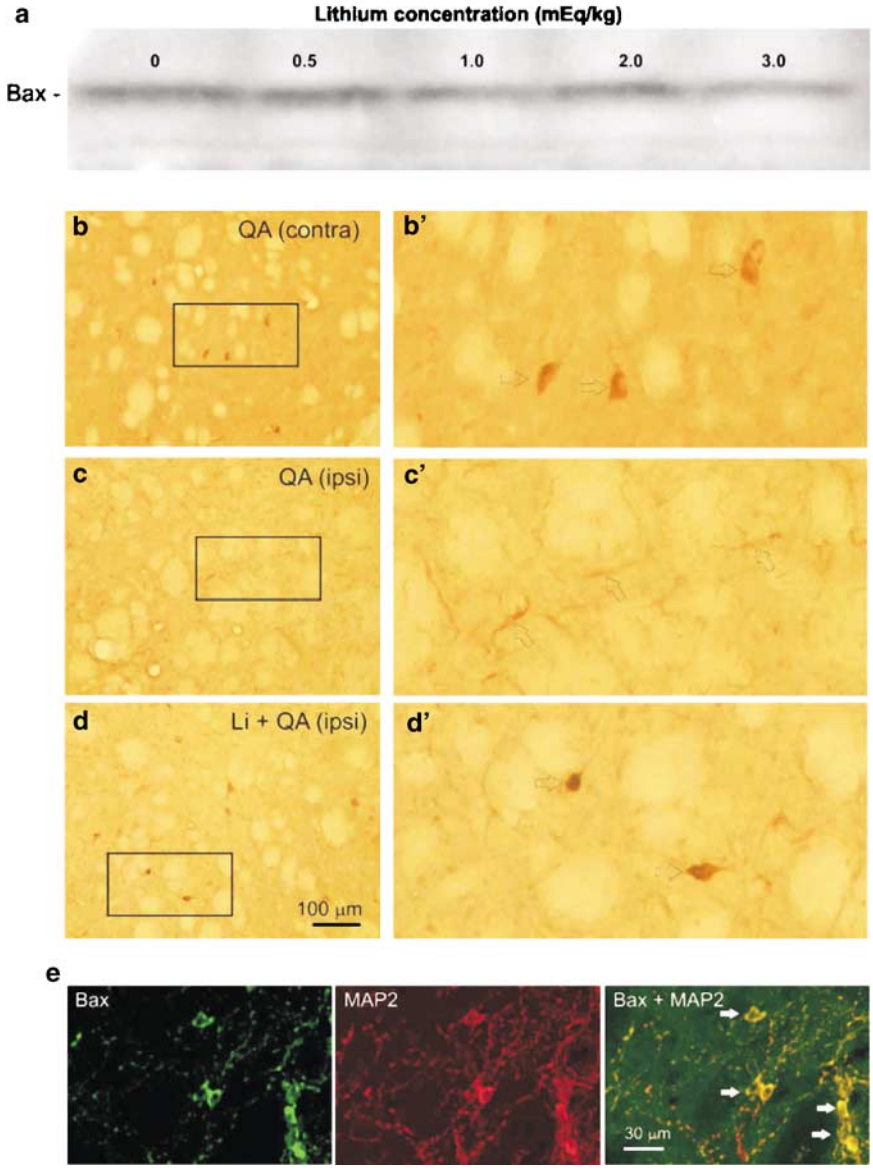

Figure 10 Bax expression in the striatum after QA injection. (a), A representative Western blot of Bax protein from the striata of QA-injected animals treated with different (0, 0.5, 1.0, 2.0 or 3.0 mEq/kg) doses of LiCl as indicated above each band. Data from one of the two experiments are shown. (b,c) Bax immunostaining of sections from contralateral (intact) and ipsilateral (injured) striata of QA-injected animals without lithium treatment ($n = 4$). (d) Bax immunostaining of section from QA-injected striatum treated with 3.0 mEq/kg of lithium ($n = 3$). Boxed regions in (b), (c) and (d) are shown under a higher magnification in (b'), (c') and (d'), respectively. Hollow arrows indicate cells with neuron-like morphology in (b') and (d') and cells with glia-like morphology in (c'). (e) Double immunostaining with antibodies against Bax and MAP2 of striatal sections from QA-injected striatum treated with 3.0 mEq/kg of lithium. Filled arrows indicate double-stained cells.

observations, we used BrdU labeling to examine the possibility that the observed increase in the number of surviving neurons in the QA-injured striatum in lithium-treated animals involved this drug-stimulated neurogenesis in the striatum. In saline-treated animals we found little, if any, BrdU labeling near the injection site, but observed substantial labeling in the narrow strip along the fourth ventricle corresponding to the SVZ. These cells were both NeuN- and GFAP negative. These observations were expected in view that the SVZ of postnatal rodents and primates contains a prolific source of neuronal progenitor cells that retain their capacity to concurrently generate neurons and migrate into the olfactory bulb, where they differentiate into neurons throughout life.[46] Unexpectedly, lithium-treated animals showed BrdU-labeled cells near the QA injection site with a concomitant loss of cells labeled with BrdU at the SVZ. While the majority of BrdU-labeled cells may represent undifferentiated progenitor cells, a small population of labeled cells was costained with anti-GFAP. This result

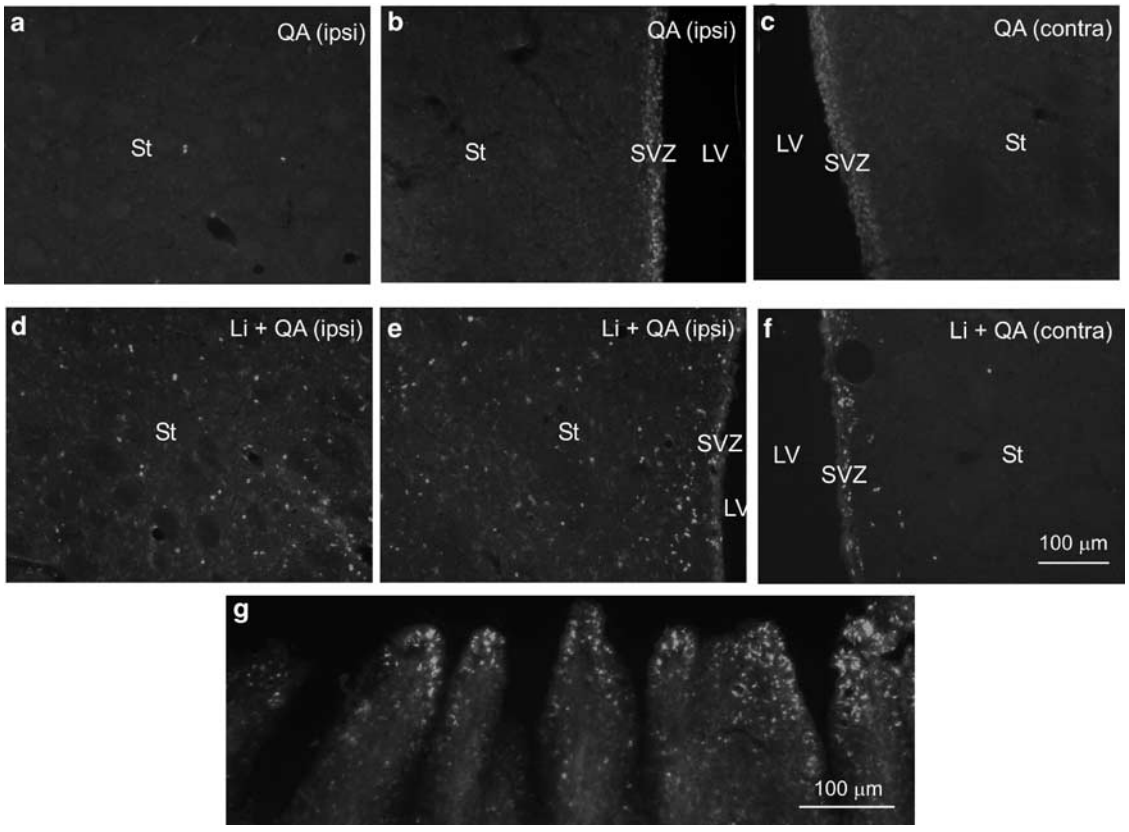

Figure 11 Lithium pretreatment induces cell proliferation in QA-injected striatum. (a–c) Sections showing BrdU labeling in the striatum at the level of QA injection in animals receiving no lithium pretreatment ($n = 3$). Panels (a) and (b) show striatal areas ipsilateral to QA injection and (c) shows contralateral striatum. Note that there are only a few BrdU-labeled cells near the QA injection site (a) but many BrdU-labeled cells in the SVZ along the fourth ventricle (b,c). (d–f) BrdU labeling of cells in the striata of rats pretreated with 3 mEq/kg of LiCl ($n = 3$) on the side ipsilateral to QA-injection (d and e) and on the contralateral side (f). Note that abundant BrdU labeling occurs near the QA injection site (d) and is associated with depletion of BrdU labeling in the SVZ (e). Also note on the contralateral side (f), the presence of BrdU-labeled cells outside SVZ. (g) Control BrdU-labeling of proliferating cells in the intestine from the same group of animals. St, striatum; LV, lateral vetricle.

may suggest that lithium stimulates astrogliogenesis in the injured striatum. Our observation of BrdU labeling of cells that express NeuN in the striatum of lithium-treated animals raises the possibility that the neuroprotective effects of lithium in the rat model of HD could be due, in part, to its ability to stimulate progenitor cell neurogenesis, migration and neuronal determination. We hypothesize that the combination of QA injury and lithium pretreatment causes neuronal progenitors migrating to the olfactory bulb to re-route into the QA-injured striatal area instead. However, we cannot exclude the possibility that the appearance of BrdU-labeled cells near the QA injection site in lithium-treated animals reflects local cell proliferation in the parenchyma of the striatum. Nor could we exclude the possibility that changes in numbers of newly divided cells and apoptosis are related. It could be possible that cells divide in the SVZ and then enter the striatum, where they rapidly turn over before differentiation. Accordingly, lithium-induced build-up of proliferating cells near the QA injection site could be due to a lower rate of apoptosis.

The mechanisms underlying this neurogenesis require further investigation but could be related to lithium's ability to increase the expression and release of brain-derived neurotrophic factor (BDNF) *in vivo* and in cultures,[16,47] leading to the activation of the BDNF receptor TrkB.[16] BDNF is a prosurvival factor produced by cortical neurons that is necessary for survival of striatal neurons, and its reduced production has been reported in the caudate and putamen of HD patients.[48] It has also been demonstrated that mutated huntingtin downregulates transcription of BDNF in HD.[49] The hypothesis that BDNF/TrkB signaling mediates lithium's effect on striatal cell proliferation is corroborated by the findings of Pencea *et al*.[50] that BDNF infusion into adult rat brain results in multiple BrdU labeling in striatal cells expressing neuronal markers and that the extent of cell proliferation in the striatum correlates with the level of TrkB expression. Our future studies will aim to substantiate the role of lithium in mediating neurogenesis and migration of newborn cells, and to study their underlying mechanisms. To date, our current results suggest that the neuropro-

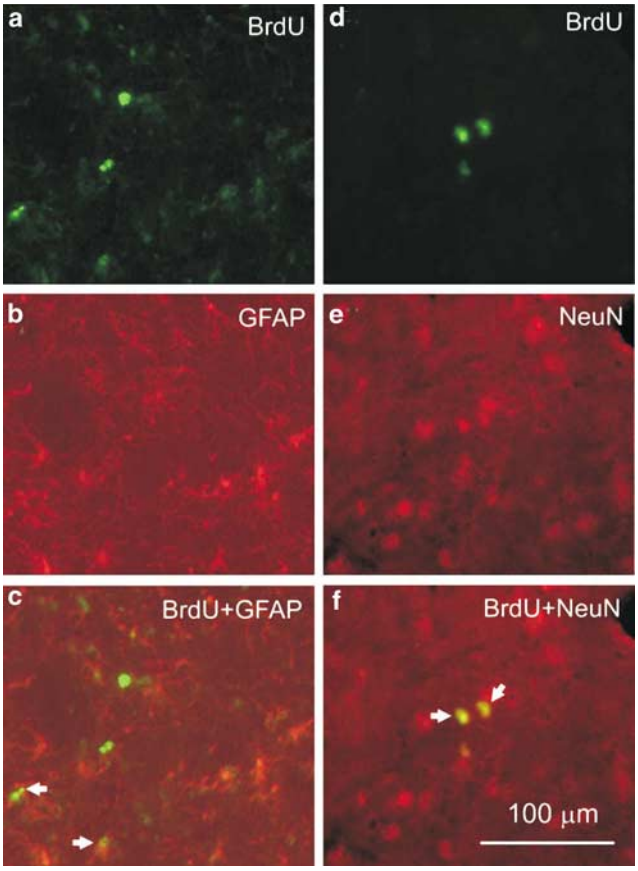

Figure 12 A small number of BrdU-labeled cells in lithium-treated, QA-infused striatum are positive for either NeuN or GFAP. (a–c) Immunostaining of BrdU-labeled cells for GFAP in lithium-pretreated, QA-infused striatum as described in the legend to Figure 11. Note that the majority of the BrdU-labeled cells are not colabeled with GFAP, and that only a weak BrdU signal was detected in colabeled cells. (d–f) Immunostaining of BrdU-labeled striatal cells for NeuN in lithium-pretreated, QA-infused animals are shown. Note that some BrdU-labeled cells are colabeled with NeuN. Arrows indicate cells double labeled for BrdU and GFAP (c) or BrdU and NeuN (f).

tective effects of lithium may involve two independent mechanisms: the prevention of apoptotic cell death and the stimulation of compensatory neurogenesis/cell migration, and indicate a potential role for the utilization of this drug in the treatment of HD.

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