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Lithium protects against oxidative stress-mediated cell death in alpha-synuclein over-expressing *in vitro* and *in vivo* models of Parkinson's disease

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Abstract

Lithium has recently been suggested to have neuroprotective properties in relation to several neurodegenerative diseases. In this study, we examined the potential cytoprotective effect of lithium in preventing oxidative stress-induced protein accumulation and neuronal cell death in the presence of increased alpha-synuclein levels *in vitro* and *in vivo*. Specifically, lithium administration was found to protect against cell death in a hydrogen peroxide treated, stable alpha-synuclein-EGFP over-expressing dopaminergic N27 cell line. Lithium feeding (0.255% lithium chloride) of 9 month old pan-neuronal alpha-synuclein transgenic mice over a 3 month period was also sufficient in preventing accumulation of oxidized/nitrated alpha-synuclein as a consequence of chronic paraquat/maneb administration in multiple brain regions including the glomerular layer, mitral cells, and the granule cell layer of the olfactory bulb (OB), the striatum, substantia nigra pars compacta (SNpc), and Purkinje cells of the cerebellum. Lithium not only prevented alpha-synuclein mediated protein accumulation/aggregation in these brain regions but also protected neuronal cells including mitral cells and dopaminergic SNpc neurons against oxidative stress-induced neurodegeneration. These results suggest that lithium can prevent both alpha-synuclein accumulation and neurodegeneration in an animal model of PD, suggesting that this drug, already FDA-approved for use in bipolar disorder, may constitute a novel therapeutic for this other human disease.

Keywords

protein accumulation; neurodegeneration; oxidized/nitrated synuclein; cytoprotection

Introduction

Lithium has been long used as a mood stabilizer for treatment of bipolar disorder, however it has recently been refocused on its possible alternative use as a novel therapeutic for various neurodegenerative conditions based on newly appreciated neuroprotective and neurotrophic properties of the drug (Pies, 2002). These include both acute brain injuries and chronic neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Sarkar et al., 2005; Feng et al., 2008; Fornai et al., 2008; Marmol, 2008). Neuroprotective effects attributed to the drug include protection against such diverse cellular disturbances as increased oxidative stress, apoptosis, inflammation, ethanol-induced neurotoxicity, and mitochondrial and endoplasmic reticulum disruption (Young et al., 2008; Machado-Vieira et al., 2009; Luo,

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2010). It has also been demonstrated to stimulate mitochondrial biogenesis and autophagy and further to increase lifespan in *C. elegans* (Fornai et al., 2008; McColl et al., 2008). Chronic lithium treatment has recently been reported to significantly attenuate neurodegeneration in the trigeminal, facial, ambiguus, and hypoglossal nuclei associated with an animal model of ALS (Ferrucci et al., 2010). Although the underlying mechanisms associated with its neuroprotective effect are not clearly understood, various molecular targets have been suggested including inositol monophosphatase (IMPase), glycogen synthase kinase-3 β (GSK-3 β), B-cell CLL/lymphoma 2 (Bcl-2), tau, extracellular signal-regulated kinase (ERK), AMP-activated protein kinase (AMPK), serine/threonine protein kinase (Akt), and glutathione S-transferase (GST) (Chen and Chuang, 1999; Chen et al., 1999; Sarkar et al., 2005; Chakraborty et al., 2008; Rametti et al., 2008; Shao et al., 2008; Young et al., 2008).

The protein alpha-synuclein has been detected in insoluble inclusions within Lewy bodies and neurites, neuropathological characteristics of PD (Spillantini et al., 1997). Neurotoxins associated with the disorder including MPTP, paraquat, and rotenone have been reported to enhance conversion of soluble alpha-synuclein to insoluble α -synuclein aggregates, contributing to neuropathology associated with PD (Przedborski et al., 2001; Thiruchelvam et al., 2002). Increased oxidative/nitrosative post-translational modification of the protein, particularly tyrosine nitration, has been suggested to enhance these effects (Przedborski et al., 2001; Gao et al., 2008). Over-expression of α -synuclein increases the sensitivity of cells to the cytotoxic damage of these neurotoxins (Richfield et al., 2002; Thiruchelvam et al., 2004; Fernagut et al., 2007). The neurotoxins, paraquat (PQ) and maneb (MB), have been demonstrated to be synergistic in terms of PD-associated neurotoxicity and their combined effect to be increased in the presence of α -synuclein expression (Thiruchelvam et al., 2002; 2004). Chronic PQ/MB administration has recently been reported to elicit both α -synuclein increase and oxidized/nitrated α -synuclein accumulation in various brain regions in a neuronal α -synuclein A53T expressing transgenic mouse model (Norris et al., 2007). Here we showed that PQ/MB-induced accumulation of oxidized/nitrated α -synuclein in A53T mutant expressing mice is attenuated by oral lithium administration. Moreover, lithium administration was also found to prevent associated neuronal cell loss in this *in vivo* model.

Materials and Methods

Cell culture and cell viability measurements; assessment of cellular autophagy

A newly established stable dopaminergic N27 cell line expressing EGFP-conjugated human α -synuclein mutant A53T cDNA was cultured in 0.5, 1.0, 2.0, or 4.0 mM LiCl or 0.4 μ M rapamycin versus 1.0 mM NaCl or untreated controls in cell culture media (10% FBS in RPMI 1640, Invitrogen, Carlsbad, CA) for 72 hours, followed by 200 μ M H₂O₂ exposure for 16 hours. Cell viability was measured by either EGFP fluorescence in similarly plated cell samples or trypan blue staining (Sigma, St. Louis, MO). Results from the latter were quantitated as percent live versus total cells (Shao et al., 2008). Cultured cells were also extracted for biochemical analyses including MTT assay (yellow tetrazolium salt, Sigma) and glutathione level measurement (ApoGSH detection kit, BioVision inc., Mountain View, CA); results were normalized per total cellular protein levels. For Western blot analysis, 20 μ g of total protein from cell extracts were loaded onto 10% SDS-PAGE gels (BioRad, Hercules, CA) and transferred to polyvinylidene fluoride (PVDF) membranes at 4 °C overnight. Rabbit polyclonal anti-LC3B primary antibody (1:1,000 dilution, NB100-2220, Novus Biologicals, Littleton, CO) and anti- β -actin antibody (1:12,000 dilution, Chemicon, Temecula, CA) as loading control were used, followed by anti-rabbit IgG and anti-mouse IgG as secondary antibody, respectively. Membranes were incubated in chemiluminescent horseradish peroxidase (HRP) substrate for 3 minutes (Thermo Scientific, Rockford, IL) before exposing to film (Kodak BioMax, Rochester, NY).

Lithium feeding and chronic PQ/MB treatment in alpha-synuclein A53T transgenic mice

All animal protocols were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals; all procedures were approved by the Buck Institute Animal Care and Use Committee. All efforts were made to minimize animal numbers and distress. Transgenic mice expressing human alpha-synuclein A53T under the control of the prion protein promoter were obtained from Jackson laboratory (Giasson et al., 2002). At 9 months of age, hemizygous transgenic littermates were fed either mouse chow (PicoLab Rodent Diet 20, Purina 5053, Richmond, IN) containing 0.255% LiCl (Lima et al., 2008) or the same chow without LiCl in age- and sex-matched siblings (n=12 males and n=10 females in each group) for a 3 month period. All the mice were then exposed to chronic PQ/MB or vehicle (saline) at 12 months of age. PQ and MB were intraperitoneally injected in a dose of 10mg/kg and 30mg/kg, respectively and twice a week for 3 consecutive weeks, as previously described (Norris et al., 2007). Weight fluctuation was monitored once a week and a few mice experiencing more than a 10% loss of body weight were excluded from the study.

Tissue extraction and Western blot analysis of total and oxidized/nitrated α -synuclein levels *in vivo*

Olfactory bulb, brain stem, striatal, cerebellar, and cortical tissues were isolated from one half of each brain for protein extraction and the other half of each brain used for immunohistochemistry (see below). All procedures for tissue extraction were performed as previously described; high salt (HS) and radioimmunoprecipitation assay (RIPA) samples were analyzed by Western blot analysis (Norris et al., 2007). For Western blot analysis, 20 μ g total proteins from each sample were loaded onto 10% SDS-PAGE gels (BioRad, Hercules, CA) and transferred to PVDF membranes at 4 °C overnight. Primary antibodies used included anti-oxidized/nitrated α -synuclein (1:1,000 dilution of mouse anti oxi/nit α -syn 514, Abcam: ab24717, Cambridge, MA), mouse anti-rat α -synuclein (1:1,000 dilution, BD Biosciences 610787, San Jose, CA), and mouse anti- β -actin (1:12,000 dilution, Chemicon, Temecula, CA) as loading control; secondary antibodies included anti-mouse IgG-HRP (1:1,000, 1:2,000, and 1:12,000, respectively). Membranes were developed as described above.

Immunohistochemistry of total and oxidized/nitrated cellular α -synuclein levels *in vivo*

For immunohistochemical analyses, brains perfused in 150 mM NaCl/70% ethanol were incubated in 30% sucrose for 2 days before embedding in OCT (optimum cutting temperature, Tissue Tek, Sakura Finetek USA, Torrance, CA); samples were kept at -80 °C until sectioning. Tissues from each experimental group were sectioned at 40 μ m using a cryostat on the same set of slides to minimize slide variability. Mouse anti-oxidized/nitrated α -synuclein (1:400 diluted Syn514, Abcam, Cambridge, MA) monoclonal or anti-human specific α -synuclein (1:500 diluted mouse anti-alpha-synuclein monoclonal, MAB 5320, Chemicon) antibodies were incubated at 4 °C overnight, followed by incubation in secondary antibodies for Syn514 (1:400 dilution, Chemicon) or for alpha-synuclein (1:200 diluted biotinylated anti-mouse IgG, Chemicon) for 90 minutes at room temperature. For quantitation of cells, mitral and Purkinje cell-specific staining using 1:200 diluted rabbit anti-5HT-2A (anti-Serotonin 2A) receptor polyclonal antibody (ab16028, Abcam) or dopaminergic-specific SNpc staining using 1:500 diluted rabbit anti-tyrosine hydroxylase polyclonal antibody (AB152, Chemicon) were used and followed by incubation in 1:200 diluted anti-rabbit biotinylated secondary antibody. Label intensity was enhanced via incubation in a 1:100 dilution of Vectastain ABC kit solution (Vector Laboratories inc., Burlingame, CA) for 60 minutes followed by development in diaminobenzidine (DAB, Chemicon).

Stereological cell count analyses

Cell type-specific labeled cells (anti-5HT-2A receptor antibody for mitral and Purkinje cells and anti-TH antibody for SNpc DA neurons) were stereologically counted using the optical fractionator method (Stereo Investigator, MBF Bioscience, Williston, VT) to quantitatively compare numbers in lithium-treated versus vehicle controls as previously described (Kim et al., 2011). All stereological cell counts were performed by a rater who was blind to treatments and are presented as mean labeled cell number $\pm$ SEM (analyzed by paired t-test, $n = 6-7$ per group).

Statistical analyses

For cell culture studies, effects of increased lithium concentrations were statistically evaluated versus 1.0 mM NaCl H_2O_2 -treated controls for significance on cell viability and GSH level (one-way ANOVA followed with the post hoc analysis Dunnett's t-test, $n=3$). For stereological analyses in Figs. 5 and 6, paired t-test was used to compare lithium-treated animals with vehicle-treated control siblings due to the presence of one measurement variable and two nominal variables (different group of animals) in these analyses. We additionally assumed that the differences between pairs are normally distributed and the mean difference between paired observations is zero in the null hypothesis. Thus, the nominal variable represents the different animals and results are presented as mean $\pm$ SEM ($n = 6-7$ per group). For all studies, $p < 0.05$ was considered statistically significant; GraphPad Prism 5 software was used for all data analyses.

Results

Lithium protects against oxidative stress-mediated cell death in the presence of alpha-synuclein A53T expression *in vitro* but not via increased autophagy

Using a stable human α -synuclein A53T over-expressing dopaminergic N27 cell line, we measured cell viability via qualitative assessment of numbers of EGFP fluorescent cells, quantitative counts of trypan blue stained cells, measurement of mitochondrial reductase activity via the MTT assay (Mosmann, 1983), and of total cellular glutathione (GSH) levels. Results from all four assays demonstrated that lithium significantly protected against cell death induced by hydrogen peroxide administration (one-way ANOVA, Dunnett's post hoc test, F-value: 56.57 for 1G, $F=15.84$ for 2A and 17.47 for 2B with d.f.=19, Figs. 1 and 2A-B). Treatment with LiCl alone (1.0 mM) showed no differences in terms of cell viability compared to 1.0 mM NaCl treatment in any of the conditions examined (data not shown). We observed that lower than 0.5 mM LiCl did not affect cell survival while doses higher than 4 mM LiCl appeared to be slightly toxic. Western blot analysis of the LC3I versus II ratio as a measure of atg 5/7-dependent autophagy was not altered by lithium treatment (Fig. 2C, Zhong et al., 2006). In contrast, while rapamycin was found to induce increases in autophagy by this assay, this was not protective against subsequent H_2O_2 -mediated cell loss (Figs. 2A-C).

Lithium attenuates PQ/MB-induced increases in total and oxidized/nitrated alpha-synuclein levels in various brain regions in A53T expressing transgenics

Hemizygous transgenic mice expressing human alpha-synuclein A53T pan-neuronally were administered either oral lithium or vehicle starting at 9 months of age (when neurodegeneration in this line is first noted) for a 3 month period (Giasson et al., 2002). At 12 months of age, mice were exposed to either chronic PQ/MB exposure or vehicle as previously described (Norris *et al.*, 2007). As lithium is known to increase potential for weight loss, we monitored animals' weights and excluded those with $>10\%$ loss from subsequent analyses (less than 10% of lithium fed mice). Olfactory bulb, brain stem, striatal,

cerebellar, and cortical tissues were isolated from one half of each brain for Western blot analyses of total and oxidized/nitrated α -synuclein levels in the various brain regions, followed by immunohistochemical analyses of cellular distribution of oxidized/nitrated α -synuclein levels in the other half. PQ/MB treatment was found to increase levels of total alpha-synuclein in all brain regions examined. However, lithium-treated transgenic mice samples from these brain regions displayed substantially lower levels of both total (16 kD or higher) and oxidized/nitrated α -synuclein in the presence of chronic PQ/MB exposure, compared with vehicle-treated controls (Fig. 3A-B). Densitometric analyses of levels of oxidized/nitrated α -synuclein normalized for β -actin were performed on triplicate Western blots including for BS (Fig. 3C), OB (Fig. 3D), Cx (Fig. 3E), Cb (Fig. 3F), and St (Fig. 3G). The lithium-treated PQ/MB group displayed significantly lower levels of oxidized/nitrated α -synuclein accumulation than the vehicle + PQ/MB group ($p < 0.05$ or 0.01 in all analyzed regions, one-way ANOVA with Dunnett's post hoc test, F-values: 15.63 (BS), 26.09 (OB), 18.28 (Cx), 17.83 (Cb), 13.17 (St) with d.f.=18). Lithium without PQ/MB treatment showed no significant differences compared with vehicle without PQ/MB in most tissues (Fig. 3). Cellular distribution of oxidized/nitrated α -synuclein levels in lithium-treated brains (Figs. 4C and D) and vehicle-no PQ/MB controls (Figs. 4E and F) versus vehicle-PQ/MB treated sibling controls (Figs. 4A and B) were compared using parallel immunohistochemical staining of samples sectioned onto the same slide to reduce slide-to-slide variation. Labeling intensity of oxidized/nitrated α -synuclein was measured in various affected brain regions including the glomerular layer and granule cell layer of the OB, the striatum, the hippocampus, SNpc, and cortex, followed by background level correction. Labeling intensities of oxidized/nitrated α -synuclein in the glomerular layer, the granule cell layer, the SNpc, and the striatum were significantly lower in the lithium-PQ/MB treated group compared to those in vehicle-PQ/MB treated controls (Fig. 4G, mean $\pm$ SEM, one-way ANOVA, the Dunnett's post hoc test, $p < 0.05$ for GL ($F=13.48$), GCL ($F=6.72$), and St ($F=7.07$), $p < 0.01$ for SNpc ($F=18.51$ with d.f.=17), $n=6$ each). The label intensity in most tissues was found to be significantly lower in lithium-treated brains versus untreated controls except for the hippocampus and cortex. Following staining for oxidized/nitrated α -synuclein, we also stereologically counted labeled mitral cells in the OB and Purkinje cells in the cerebellum which, due to their distinct anatomical distribution, we could easily identify (Figs. 5A-C, vehicle; Figs. 5D-F, lithium). While those neurons were consistently more strongly labeled in the untreated controls, we rarely identified oxidized/nitrated α -synuclein labeled cells in tissues isolated from lithium-treated and no PQ/MB injected animals; this was verified by subsequent stereological analyses (Fig. 5G). For example, we counted oxidized/nitrated α -synuclein-labeled mitral cells (mean $\pm$ SEM, no lithium + PQ/MB: 7635.2 ± 1190.8 versus Li + PQ/MB: 1430.4 ± 351.3) and oxidized/nitrated α -synuclein-labeled Purkinje cells (no lithium + PQ/MB: 8792.0 ± 3571.5 vs. Li + PQ/MB: 1568.0 ± 495.5). In addition, since we did not observe significant differences in labeled cell numbers between lithium + PQ/MB and no lithium - PQ/MB group, we did not include no PQ/MB controls in the analysis of paired t-test (data not shown). We found on average that only 18.73% of mitral cells and 17.83% of Purkinje cells displayed oxidized/nitrated α -synuclein accumulation in the presence of lithium compared with no lithium controls (100%, $n=6-7$ per group, paired t-test: $p < 0.01$). We found no oxidized/nitrated α -synuclein positive cells in mitral and Purkinje cells from no lithium-no PQ/MB controls.

Lithium prevents PQ/MB induced cell loss in various brain regions of A53T transgenics

Lastly, we quantitated the impact of lithium treatment on preventing PQ/MB-induced neuronal cell loss in A53T hemizygous transgenics using cell-type specific markers, anti-5HT-2A receptor antibody for mitral cells (Figs. 6A and D) and Purkinje cells (Figs. 6B and E) and anti-TH antibody for dopaminergic neurons in the SNpc (Figs. 6C and F). Results from stereological counts demonstrated that neuronal mitral cell loss (mean $\pm$ SEM,

no lithium + PQ/MB: 7756.7 ± 767.4 vs. lithium + PQ/MB: 11166.0 ± 474.9 , Fig. 6G, $p < 0.01$) and TH-positive dopaminergic SNpc neuronal loss (mean $\pm$ SEM, no lithium + PQ/MB: 6919.6 ± 305.3 vs. lithium + PQ/MB: 7897.0 ± 346.3 , Fig. 6I, $p < 0.05$) were significantly prevented by lithium, while numbers of labeled Purkinje cells were not significantly different between the two groups (mean $\pm$ SEM, no lithium + PQ/MB: 42414.0 ± 955.2 vs. lithium + PQ/MB: 46211.3 ± 701.9 , Fig. 6H, paired t-test, $n=6$ per group, Fig. 6H). Since we observed no significant differences in cell numbers between lithium-PQ/MB treatment and lithium-no PQ/MB controls, we did not include the analysis of no PQ/MB controls in the paired t-test (Figs. 5 and 6).

Discussion

Lithium administration *in vitro* was found to protect against hydrogen peroxide-induced cell death in a stable human alpha-synuclein-expressing dopaminergic N27 cell line. Lithium's ability to prevent reductions in GSH levels could be in part due to its previously reported ability to protect against losses in glutathione S-transferase (GST) activity as well as via general cell protective effects (Shao et al., 2008). Lithium's *in vitro* neuroprotective effects do not appear to be due to lithium's reported ability to increase cell autophagy (Sarkar et al., 2005); conversely while rapamycin did induce increased autophagy, this did not elicit neuroprotective effects in our cellular PD model. Levels of apoptosis as measured via an Apo 3/7 caspase activity assay following lithium treatment in our cell model displayed a trend towards reduction, although differences were not consistently significant (data not shown). We also found no significant changes in levels of either GSK-3 β phosphorylation or bcl-2 that could explain lithium's neuroprotective effects in our cell model. Microarray analysis performed as a non-biased means of detecting genes whose expression were changed by lithium treatment also did not reveal any novel candidates for its neuroprotective effects (data not shown).

We tested the cytoprotective effect of lithium against PQ/MB in α -synuclein A53T over-expressing mouse model because A53T mutant formed Lewy-body like fibrils and discrete spherical assemblies at higher concentrations and most rapidly, compared with wild type and A30P mutant forms of α -synuclein. Multiple studies demonstrated that A53T is more sensitive in terms of forming protein aggregates than other mutant forms including A30P and wild type α -synuclein (Conway et al., 1998; Serpell et al., 2000). Thus, we used A53T over-expressing transgenics for testing cytoprotective effect of lithium in preventing protein aggregation in this study. Although we have not identified PD-related symptoms from any of those alpha-synuclein A53T over-expressing hemizygous mice at the age of 12 month old, we confirmed previous *in vivo* studies demonstrating that chronic PQ/MB administration elicits both α -synuclein increase and oxidized/nitrated α -synuclein accumulation in various brain regions in a neuronal α -synuclein A53T expressing transgenic mouse model (Norris et al., 2007). Levels of both were found to be significantly reduced in the presence of lithium treatment. Furthermore, three months of lithium feeding was sufficient to protect PD-sensitive neurons including mitral cells of the OB and dopaminergic neuronal cells in the SNpc against subsequent PQ/MB-induced cell loss, while no significant differences were found in Purkinje cell numbers even though oxidized/nitrated α -synuclein accumulation was prevented in these cells. In addition, we did not find the potential protective effect of α -synuclein in OB and SNpc against PQ/MB observed in the previous report and in fact found higher levels of oxidized/nitrated α -synuclein accumulation following PQ/MB exposure. The discrepancies between this study and the previous report may be explained by the fact that we used hemizygous transgenics in this study and did not observe the behavioral symptoms reported at 12 months in the homozygous mice used in the previous report (Norris et al., 2007).

We also observed no changes in previously reported targets of lithium feeding that would explain its neuroprotective effects in the *in vivo* mouse model used in this study, including phosphorylated GSK-3 β , LC3II, or Bcl-2 (data not shown, Chalecka-Franaszek and Chuang, 1999; Sarkar et al., 2005; Pasquali et al., 2010). Several reports have suggested that lithium neuroprotection may occur by effects separate from these previously identified targets; for example, lithium protects cerebellar granule cells from ethanol-induced apoptosis in a manner independent of GSK-3 β (Zhong et al., 2006) or its phosphorylation levels following chronic lithium exposure (Yuskaitis et al., 2010). It is also possible that macroautophagy may occur in a manner independent of Atg 5/7 which would therefore not induce increases in the LC3II/I ratio (Nishida et al., 2009). Based on quantitative PCR and microarray results using N27 cell extracts, we found no α -synuclein mRNA down-regulation, thus lithium likely degrades aggregated alpha-synuclein and/or prevents oxidized/nitrated alpha-synuclein accumulation *in vivo* post-translationally. These results support the alternative possibility that lithium may increase proteasomal activity which would explain lithium-mediated reductions in both total α -synuclein and oxidation/nitration levels that we observed in various brain regions in the *in vivo* model (Ferrucci et al., 2008). The potential underlying mechanism(s) of lithium's neuroprotective effects in the context of this model remains to be elucidated and is currently under continuing investigation in the laboratory. However, in a recent clinical human pilot study, lithium feeding was found to delay ALS disease progression through multiple pathways including autophagy, biogenesis of mitochondria, neurogenesis, and neuronal differentiation (Fornai et al., 2008), although in a subsequent human ALS multicenter trial lithium carbonate showed a lack of efficacy (Chio et al., 2010). Efficacy may be dependent on several variables including compound formulation, dosage and delivering methods and therefore understanding the underlying mechanisms involved in lithium's neuroprotective effects will be a paramount next step in these ongoing studies.

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Fig. 1. Lithium protects against H₂O₂-induced cell death in a stable A53T alpha-synuclein-EGFP over-expressing dopaminergic N27 cell line. Protective effects of increasing concentrations of LiCl (0.5-4.0 mM) compared to NaCl-treated controls (1.0 mM) on H₂O₂-induced cell loss as displayed by EGFP fluorescence (A-F). Cell viability is quantitated by counts following trypan blue staining (G); values are reported mean $\pm$ SEM compared to 100% control (n = 3 each); **, p < 0.01 versus 200 μ M H₂O₂-treated NaCl controls as assessed by one-way ANOVA, the post hoc analysis Dunnett's t-test.

Fig. 2.

Lithium protects against H₂O₂-induced cell death in alpha-synuclein A53T expressing dopaminergic N27 cells as assessed by MTT assay (OD_{570/630}) (A) and total glutathione levels (a.u.: arbitrary unit) (B). Values reported as mean $\pm$ SEM; *, $p < 0.05$ and **, $p < 0.01$ compared with 1.0 mM NaCl, H₂O₂-treated controls (one-way ANOVA, the Dunnett's post hoc test). LC-3II Western blot analysis of autophagy (C); LiCl-treatment versus NaCl and Rap controls; β -actin is a loading control. Rap, 0.4 μ M rapamycin.

Fig. 3.

Lithium prevents oxidized/nitrated alpha-synuclein accumulation (oxi/nit α-Syn, 16 kD) in multiple brain regions of neuronal human A53T mutant expressing mice chronically exposed to PQ/MB. Examples of Western blot analysis of high salt (A) and RIPA (B) soluble extracts are displayed and densitometric analyses in oxi/nit α-syn Western blots shown in BS (C), OB (D), Cx (E), Cb (F), and St (G) of high salt samples, normalized for β-actin loading. *, $p < 0.05$, **, $p < 0.01$ versus vehicle with PQ/MB (black bars) as assessed by one-way ANOVA, the Dunnett's post hoc test ($n = 4-6$ per group). Abb.: BS, Brain stem; OB, olfactory bulb; Cx, cortex; Cb, cerebellum; St, striatum; Li, lithium treatment; PQ/MB, paraquat/maneb treated group.

Fig. 4. Lithium prevents oxidized/nitrated alpha-synuclein accumulation in multiple brain regions. Examples are shown of OB (A, C and E) and striatum (B, D and F). Mean density $\pm$ SEM after background correction (a.u.: arbitrary unit) (G). One-way ANOVA, the Dunnett's post hoc test ($n=6$ each); *, $p<0.05$, **, $p<0.01$, n.s., not significant compared to no lithium group + PQ/MB (black bars). Cont: no lithium without PQ/MB injection. Size bar: 250 μ m. Abb.: GL, glomerular layer; GCL, granule cell later; St, striatum; SNpc, Substantia Nigra pars compacta; HP, hippocampus; Cx, cortex.

Fig. 5. Lithium prevents oxidized/nitrated alpha-synuclein accumulation in mitral (red arrows in A and D) and Purkinje cells (red arrows in B and E). A, B, and C: vehicle-treated group; D, E, and F: lithium-treated group. C and F are enlargements of B and E, respectively. G: Oxidized/nitrated alpha-synuclein positive cells were stereologically counted and reported as mean $\pm$ SEM (n=6-7 each). **, $p < 0.01$ versus controls as assessed by paired t-test. Bar: 75 μ m.

Fig. 6.

Lithium protects against neuronal cell death in several brain regions. Mitral cells (A and D) and Purkinje cells (B and E) were specifically labeled with anti-5HT-2A receptor antibody and SNpc DA neurons (C and F) with anti-TH antibody. Total cell numbers were stereologically counted following specific cell labeling (G, mitral cells; H, Purkinje cells; I, SNpc DA cells) and reported as mean $\pm$ SEM (n=6-7 each). *, $p < 0.05$, **, $p < 0.01$ versus vehicle control as assessed by paired t-test. Bar: 100 μ m.