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Lithium prevents parkinsonian behavioral and striatal phenotypes in an aged parkin mutant transgenic mouse model

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Abstract

Lithium has long been used as a treatment for the psychiatric disease bipolar disorder. However, previous studies suggest that lithium provides neuroprotective effects in neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease. The exact mechanism by which lithium exerts these effects still remains unclear. In the present study, we evaluated the effects of low-dose lithium treatment in an aged mouse model expressing a parkin mutation within dopaminergic neurons. We found that low-dose lithium treatment prevented motor impairment as demonstrated by the open field test, pole test, and rearing behavior. Furthermore, lithium prevented dopaminergic striatal degeneration in parkin animals. We also found that parkin-induced striatal astrogliosis and microglial activation were prevented by lithium treatment. Our results further corroborate the use of this parkin mutant transgenic mouse line as a model for PD for testing novel therapeutics. The findings of the present study also provide further validation that lithium could be re-purposed as a therapy for PD and suggest that anti-inflammatory effects may contribute to its neuroprotective mechanisms.

Keywords

Parkinson's disease; lithium; striatum; neuroinflammation; behavior; astrocyte

1. Introduction

Parkinson's disease is a common neurological movement disorder characterized by bradykinesia, muscle rigidity, and resting tremor. The main pathological feature of PD is loss of dopamine nigrostriatal neurons. Although the exact etiology of neurodegeneration in PD is unclear, neuronal cell loss has been linked to protein mutations, oxidative stress, environmental toxins, and neuroinflammation (Reeve et al., 2014). Currently available treatments using dopamine replacement therapy can effectively ameliorate symptoms. However, chronic use of these treatments does not provide neuroprotection and can lead to

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disabling drug-induced side effects (Smith et al., 2012). Development of novel treatments for PD requires extensive preclinical testing for safety and efficacy prior to making them widely available to the patient population. Therefore, a medication that provides neuroprotection and is already approved for clinical use would be an ideal candidate for PD therapy.

Lithium is a drug approved for treatment of psychiatric diseases, primarily bipolar disorder. Lithium is able to significantly decrease manic episodes and suicidal tendencies. To date, the mechanism(s) by which lithium elicits anti-psychiatric effects is not entirely clear. However, studies indicate that lithium is a multifaceted drug that modulates multiple second messenger signaling systems, gene expression, and neurotransmission (Malhi et al., 2013).

More recently, *in vitro* and *in vivo* studies have demonstrated that lithium has neuroprotective effects in various neurodegenerative diseases. Lithium has been reported to reduce brain pathology and recovery time in experimental intracerebral hemorrhage models (Kang et al., 2012). Lithium treatment also has been demonstrated to inhibit beta-amyloid peptide production in Alzheimer's disease transgenic mice (Su et al., 2004) and to reduce tau hyperphosphorylation (Munoz-Montano et al., 1997). Striatal lesions in the rat, which mimic neurodegeneration associated with Huntington's disease, are found to be attenuated by chronic lithium exposure (Wei et al., 2001). In parkinsonian mice, low-dose lithium prevents both nigrostriatal degeneration and dopamine depletion (Kim et al., 2011; Li et al., 2013; Youdim and Arraf, 2004). *In vitro*, lithium can prevent the neurotoxic effects of MPP⁺ and rotenone, two agents linked to PD degeneration (King et al., 2001). These studies provide evidence that lithium could possibly be re-purposed as a treatment for neurodegenerative diseases.

To further validate findings from previous reports in a chronic disease model and to identify neuroprotective mechanisms associated with lithium, we studied the effects of low-dose lithium in an aged transgenic mouse model that expresses a parkin mutation selectively within dopamine neurons (Lu et al., 2009). The results of our study show that low-dose lithium can prevent parkin-induced motor impairment and striatal degeneration. Furthermore, parkin-related glial cell activation is also attenuated with lithium treatment. This suggests that lithium may be a viable option for PD and could act in part via its ability to reduce neuroinflammation.

2. Results

Mice in these studies (parkin mice and wildtype littermate controls) were treated with either normal chow or chow containing 0.125% lithium chloride, constituting ~25% of the lowest normal clinical dose in humans (0.2 mM sera versus 0.8 mM sera equivalents). Lithium feeding was commenced at 13 months of age and continued over a 10 month period. Higher long-term clinical dosages in humans (1.5–2.0 mM sera equivalents) have been linked in some patients to side-effects including acute encephalopathy, nephrogenic diabetes insipidus, and hyperthyroidism (Shen et al., 2007). Based on behavioral and neuropathological analyses, we noted no evidence of CNS effects associated with this lower dosage of the drug; in humans lowering of dosage is reported to result in disappearance of

CNS symptoms. Additionally, mice experienced no signs indicating presence of peripheral side effects (weight loss, diarrhea, excess urination, etc).

2.1. Lithium prevents parkin-induced behavioral impairment

As previously reported (Lu et al., 2009), mice expressing a truncated parkin mutation associated with reduced protein solubility and function show behavioral dysfunction analogous to parkinsonism by 16 months of age. In the present study, we analyzed the number of floor plane moves, a parameter that was previously reported to be significantly reduced in this model. We found a significant reduction in the number of floor plane moves in animals expressing the parkin mutation when compared to non-transgenic (nTg) littermate controls. This impairment was prevented by low-dose lithium administration over a period of 10 months (Fig 1A). Results from the pole test show that parkin animals take longer to descend the length of the pole. Lithium was also able to attenuate this behavior (Fig 1B). We found no major differences in turning down behavior amongst the groups (data not shown). Finally, rearing behavior in parkin animals also decreased, an additional indicator of motor impairment previously demonstrated to be reduced in these animals (Lu et al., 2009). Rearing impairment was also prevented by lithium treatment in this parkin mouse model (Fig 1C). No significant effects were noted in any of these parameters at earlier time points (i.e. after 3 months of lithium treatment, data not shown). In addition, lithium treatment in the nTg group showed no motor behavioral differences compared to the control group. Taken together, these behavioral results demonstrate that motor dysfunction associated with this particular parkin mutation can be prevented by chronic low-dose lithium treatment.

2.2. Lithium attenuates parkin-induced striatal degeneration

We found that expression of mutated parkin in these animals resulted in loss of tyrosine hydroxylase (TH) expression in the striatum (ST), similar to levels previously reported by Lu and colleagues (Fig 2A,B). Moreover, lithium treatment significantly prevented striatal TH loss in these animals. Lithium in nTg animals also produced a modest increase in TH when compared to the control group. These data suggest that lithium can prevent previously reported parkin-induced dopaminergic striatal degeneration in this model.

2.3. Lithium prevents parkin-induced neuroinflammation

Expression of glial fibrillary astrocytic protein (GFAP) has been used extensively to determine the extent of astrogliosis in mouse models of PD e.g. (Mallajosyula et al., 2008). GFAP has previously been demonstrated to be elevated in effected tissues from PD patients and, in earlier *in vitro* studies, lithium was shown to inhibit 6-OHDA-mediated GFAP elevations and subsequent neurodegeneration (Su et al., 2012; Wang et al., 2013). Based on these previous findings, we examined levels of GFAP-positive cells in the striatum of parkin mutants versus controls and if levels were reduced by lithium treatment. Animals expressing the parkin mutation had significantly more GFAP⁺ cells present in the striatum (Fig 3A,B). Furthermore, treatment with lithium reduced striatal astrogliosis to values similar to control groups.

Levels of microglial activation, another important hallmark of the disease, were also examined via measurement of striatal ionized calcium-binding adapter molecule 1 (Iba1)-

positive cells (Beraud et al., 2013; Kaushik and Basu, 2013). As in the case of striatal astrocytes, levels of activated microglia in the striatum were also found to be elevated in the parkin mutants versus controls and to be significantly reduced in the presence of lithium treatment (Fig 3C,D). Taken together, these data suggest that lithium may have potent anti-inflammatory properties in the parkinsonian brain.

3. Discussion

PD continues to be one of the most common neurodegenerative diseases. Although progress towards symptomatic treatment has been successful, prevention of disease onset and progression remains a challenge. Treatments that are already approved for clinical use can rapidly advance our ability to prevent PD. Lithium has been successful in the treatment for psychiatric and, more recently, in preclinical therapy for neurodegenerative diseases. In the present study, we provide further preclinical evidence that lithium may potentially be repurposed for PD neuroprotective therapy in a chronic age-related model of the disease. Given that aging is the number one risk factor for the disorder, this may provide a more accurate chronological model. For these studies, we utilized a transgenic mouse that closely mimics PD pathophysiology. Parkin, an E3 ligase, plays an important role in the degradation of targeted proteins. Mutations in parkin are present in familial forms of PD. As previously reported (Lu et al., 2009), we show that this particular transgenic mouse model, which expresses mutated parkin in dopamine neurons associated with reduced parkin solubility and E3 ligase function, displays open field motor dysfunction and rearing impairment. Novel to this model, we additionally show that it also has impairments in the pole test. We also confirm striatal axonal degeneration in this model, another feature found in PD and other parkinsonian models.

Astrocytes and microglia play an important role in brain homeostasis. They provide neurotrophic nutrients, neurotransmission efficacy, biochemical balance, and immune defense. However, insult to the brain can elicit a negative response by activation of these cell types especially if chronic, leading to numerous detrimental effects including continued release of cytokines and reactive oxygen and nitrosative species (ROS, RNS) which can produce neuronal damage. Exposure to the PD-inducing toxin 6-hydroxydopamine results in increased astrocytic and microglial activation along with expression of indices of RNS including inducible nitric oxide (iNOS) and nitrite (Wang et al., 2013) (Stott and Barker, 2014) (Hernandez et al., 2013); lithium was found to result in decreased glial cell activation. Increased glial cell activation is known to occur in affected tissues from PD patients (Mythri et al., 2011; Su et al., 2012) (Richardson and Hossain, 2013). In our study, we demonstrate that low-dose lithium prevents increased striatal neurodegeneration *in vivo* in a parkin mouse model. Interestingly, parkin knockout alone in mice does not induce nigrostriatal degeneration or cause motor defects (Frank-Cannon et al., 2008). Parkinsonism was only achieved through additional inflammatory insult induced by lipopolysaccharide (LPS) administration. Lithium has previously been reported to have neuroprotective effects in acute *in vivo* toxin PD models (MPTP; Youdim and Arraf, 2004; paraquat-treated alpha-synuclein transgenics, Kim et al., 2011). In contrast to these models, the parkin mutant model displays age-related neurodegenerative effects in the absence of the need for additional toxin exposure that are reversible by lithium treatment, thus allowing the affects

of both age and genetics to be assessed. Results in our parkin mutation model moreover demonstrate presence of neuroinflammation in the absence of additional inflammatory stressors, as occurs in the human disease itself. Taken together, this suggests that this particular parkin mutant transgenic constitutes an effective model for studying the effects of novel potential therapeutics on parkinsonian motor behavior, striatal dopaminergic loss, and associated neuroinflammation.

In contrast to the previous report from Lu and colleagues using this model in which they reported loss in SN DAergic neurons by 16 months of age, we did not observe a significant cell loss via stereological analysis at either 16 or 20 month time points (data not shown). Observed deficits in motor behavior may be a consequence of observed reductions in striatal TH levels, which could indicate neurite degeneration in the absence of frank cell loss. The neuroprotective effects of lithium may be in part due to its ability to prevent axonal degeneration mediated via neuroinflammatory processes. It may alternatively or additionally be acting via direct protective effects on the neurons by restoring ST TH and subsequent DA levels which could also contribute to the abrogation of motor defects. Lithium pre-treatment of dopaminergic cells has been reported to increase TH expression and resulting in elevations in DA release (Baker et al., 2000; Willing et al., 2002; Brandish et al., 2005; Arraf et al., 2012).

Another major debilitation experienced by PD patients are several non-motor symptoms associated with the disease, including cognitive and psychiatric problems (Smith et al., 2012). In models of Alzheimer's disease characterized by memory loss and cognitive instability, lithium has been shown to be effective in reversing these symptoms (Munoz-Montano et al., 1997; Su et al., 2004). This unique ability for lithium to prevent cognitive decline in the context of neurodegeneration has further important implications for its use as a neuroprotective agent for PD. Future studies examining the effects of lithium will be necessary in order to explore effects of the drug on such non-motor symptoms in relation to PD.

4. Experimental Procedures

4.1. Animals

Heterozygous mice expressing a mutated parkin gene under the dopamine transporter promoter were utilized for this study. Description of these mice is detailed in a previous report (Lu et al., 2009). Animals were genotyped to validate expression of the transgenic parkin mutation. Non-transgenic (nTg) mice not expressing the mutation were used as negative litter-mate controls. Animals were aged to 13 months and then treated with either lithium (chow containing 0.1275 % lithium chloride) (Kim et al., 2011) or normal chow *ad libitum* for up to 10 months of age (N= 6–8 per group). Final age of mice was 23 months. Behavioral effects were measured following 3 versus 10 months of lithium treatment; immunocytochemical parameters were measured at the time of sacrifice (following 10 months of lithium administration). Animals had free access to water and were kept on a 12 hr light/dark cycle. All animal protocols were monitored and approved by the Buck Institute Institutional Animal Care and Use Committee (IACUC). Experiments were conducted

according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1978).

4.2. Open Field Test

To evaluate spontaneous movement, animals were placed individually in a Tru-Scan open field apparatus for 10 minutes and allowed to freely explore the chamber as previously described (Lieu et al., 2013; Lu et al., 2009). Open field parameters were generated via Tru-Scan computer software. At the end of the testing period, animals were removed from the chamber and returned back to group housing. Two trials were taken then averaged.

4.3. Pole Test

To test motor initiation and coordination, animals were placed individually on top of a rough-surfaced pole head up (Fleming et al., 2004; Lieu et al., 2013). We first evaluated the amount of time for the animal to turn its head down for initial descent. We then evaluated the amount of time to reach the bottom of the pole. Each animal was tested for five trials. All trials were video recorded and the best performance was taken. Animals were trained and habituated on the pole the day prior to testing. Animals were placed back into group housing after testing.

4.4. Cylinder Rearing Test

To evaluate spontaneous motor initiation, animals were placed individually in a cylinder to determine the number of rearing events over 45 seconds as described in previous reports (Fleming et al., 2004; Lu et al., 2009). Each rearing session was video recorded. The average number of rears over two trials was taken. After testing, animals were returned back to group housing.

4.5. Immunohistochemistry

At the end of behavioral testing, animals were euthanized and brains harvested for immunohistochemistry. Brains were fixed in 4% PFA then incubated in 30% sucrose. Brains were kept frozen then embedded in OCT and sectioned at 40 μ m on a cryostat. Brain sections were stained for GFAP to evaluate astrocyte proliferation and with Iba1 to assess microglial activation (increase in cells with amoeboid morphology). Previous reports have shown increased GFAP expression in parkinsonian brains, indicating presence of astrogliosis. Brain tissue was incubated in blocking buffer for 60 min. After washing, the tissue was incubated overnight at 4°C in anti-GFAP-Cy3 primary antibody (Sigma-Aldrich, Cat#: C9205, 1:400). After washing, tissue was mounted onto slides then coverslipped with mounting media. GFAP-positive cells were counted using methods previously described (Cerbai et al., 2012; Mallajosyula et al., 2008).

Microglial activation was detected using primary antibody against Iba1 (Wako, 1:500) as described previously (Peng et al., 2009). Briefly, the sections were incubated with primary antibody in blocking buffer at 4 °C overnight, followed by incubation with Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) secondary antibody for 60 min at room temperature. After washing in TBS with 1% Triton X-10, the sections were coverslipped with permanent mounting media containing DAPI.

Representative striatal regions of interest (N = 3 animals per group) were captured at 40x objective. Cells were then counted using ImageJ software. To measure dopaminergic striatal degeneration, representative striatal sections (N = 3–4 animals per group, total of 10 representative striatal sections per animal) were taken to analyze TH striatal density using methods previously described (Kim et al., 2011). Striatal sections were washed then blocked with 5% goat serum for 30 min at room temperature. Sections were then incubated with anti-tyrosine hydroxylase (Millipore, Cat#: AB152, 1:1000) overnight at 4°C. Sections were washed and incubated with goat x rabbit IgC Fc Biotin (Chemicon, Cat#: AP156B, 1:2000). Sections were washed then processed using the ABC Elite Standard Vectastain solution (Vector Labs, Cat#: PK-6100) and exposed to DAB (Vector Labs, Cat#: SK4100). Tissue was then mounted onto slides, incubated in ascending alcohol, xylene and coverslipped. Striatal density was analyzed using ImageJ with the corpus callosum used for background correction.

4.6. Statistics

Two-way analysis of variance (ANOVA) with post-hoc Fisher's LSD multiple comparisons tests was utilized (Graphpad Prism Statistical Software). Data are expressed as mean $\pm$ SEM. We set significance to $p < 0.05$.

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Highlights

1. Lithium prevents motor impairment in the parkin mutant model of Parkinson's disease.
2. Lithium prevents dopaminergic striatal degeneration in the parkin mutant mouse model.
3. Lithium inhibits astrogliosis in the parkin mutant striatum.

Fig. 1.

Lithium prevented parkin-induced (A) impairment of floor plane moves in the open field test, * $p < 0.05$ compared to other groups, (B) pole test motor coordination, and (C) rearing movements. * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Lithium prevented axonal degeneration in the parkin transgenic mouse

A) Representative images of striatal tyrosine hydroxylase (TH) immunohistochemistry from animals in each group are shown (A- nTg mice on control diet, B- parkin mice on control diet, C- nTg fed with lithium and D- parkin fed with Lithium).

B) Quantitation of optical density of TH-IR innervation in the striatum (TH STR). The values are average striatal intensity from 3 to 5 sections per animal. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. Lithium inhibits glial activation in the parkin mouse

A. Representative images of GFAP staining in striatum sections from (A) nTg mice and (B) parkin mice with and without Lithium.

B. Quantitation of GFAP positive cells in striatum. *** $p < 0.001$, **** $p < 0.0001$.

C. Representative photomicrographs of Iba1 immunostaining in Striatum. In nTg mice fed with control (A) and lithium diet (C), microglia is at the resting stage with a small rod shaped soma and ramified processes. Numerous activated microglia showing round shaped cell body with blunt or no processes were found in striatum of parkin mice (B). Lithium treatment significantly reduced the number of activated microglia in the striatum of parkin mice (D).

D. Quantitation of the activated microglia in the striatum. *** $p < 0.001$, **** $p < 0.005$.