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Iron and Lactoferrin Levels Remain Unchanged in Parkin Deficient Mouse Brains: Implications for Parkinson's Disease Etiology

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biochemistry & Molecular Biology

By

Hanan Baker

2015

ABSTRACT OF THE THESIS

Iron and Lactoferrin Levels Remain Unchanged in Parkin Deficient Mouse Brains: Implications for Parkinson's Disease Etiology

By

Hanan Baker

Master of Science in Biochemistry & Molecular Biology

University of California, Los Angeles, 2015

Professor Jorge Torres, Chair

Parkinson's disease is a devastating neurodegenerative disease in which loss of dopaminergic neurons in the substantia nigra pars compacta leads to a variety of motor and non-motor deficits. Mutations in the Park2 or parkin gene have been implicated in familial forms of Parkinson's disease. Parkin is an E3 ubiquitin ligase that plays a role in mitophagy, the degradation of damaged mitochondria. Defects in parkin are thought to impair mitophagy, leading to mitochondrial dysfunction and an increase in oxidative stress. Although best known for its participation in the regulation of mitochondrial dynamics, parkin has also been known to play a role in cell cycle progression. My lab had previously found that parkin binds to and selectively ubiquitinates lactoferrin in human cells. Lactoferrin is an iron binding protein predominantly found in milk and implicated in several beneficial processes. In this study I further evaluated this novel interaction in a parkin knockout mouse model. I found that parkin does indeed bind lactoferrin in mouse brains, however, knockout of parkin does not cause any change in lactoferrin or iron levels.

The thesis of Hanan Baker is approved

Marie-Francoise Chesselet

Albert Courey

Jorge Torres, Committee Chair

UNIVERSITY OF CALIFORNIA LOS ANGELES

2015

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta, resulting in a deficiency of DA in the striatum. PD manifests itself in a variety of motor and non-motor symptoms such as bradykinesia, rigidity, and tremor as well as olfactory impairments, gastrointestinal dysfunction, depression, sleep disturbances, and cognitive impairments [1].The exact cause of the degeneration of the dopaminergic nigrostriatal pathway in PD remains unknown, but it is thought to be a combination of complex interactions between genetic and environmental factors. Several possible mechanisms have been postulated in addition to genetic factors including mitochondrial dysfunction, oxidative damage, abnormal protein accumulation, inflammatory processes, and other environmental toxins [2].Either alone or in combination these factors are able to cause altered synaptic plasticity, morphological changes, and eventually cell death.

Pathologically, PD is defined by the presence of Lewy bodies, amyloid inclusions primarily made up of α-synuclein [3]. α-synuclein is an intrinsically disordered protein with unknown function that under physiological conditions will assemble into aggregates similar to those found in the inclusions of diseased brains [4]. In addition, point mutations in the α-synuclein encoding gene have been shown to cause rare familial forms of PD [5]. The mutant forms of α-synuclein are more prone to misfolding, which strongly suggests that protein misfolding contributes to the formation of toxic species. The simplicity of this amyloid hypothesis has made it an attractive candidate as the underlying cause of PD. The amyloid hypothesis also brings up a possible mechanism for spreading of the toxic species in a prion-like manner. This is supported by experimentation showing that fibrillar α-synuclein is able to spread through cells and seed

further aggregation [6]. In further support of this theory, the progression of α-synuclein pathology correlates with the staging protocol for classifying brain pathology in sporadic PD [7].

Another possibility for the cause of PD that has gained momentum in recent years is that long term exposure to pesticides can increase the likelihood of the disease. There is an established association between PD and exposure to certain risk factors such as living in a rural area, well water use, farming, exposure to farm animals, and exposure to pesticides [8]. In fact, individuals exposed to pesticides showed a 70% higher incidence of PD than those not exposed [9]. Although in general pesticide exposure has been shown to be linked to PD, only a few specific chemicals have been confirmed and further studied. Among those, the most prominent are ziram, maneb, and paraquat. At workplaces, combined exposure to these pesticides increased the risk of PD three-fold, suggesting that several mechanisms may work in conjunction to cause the disease [10].

Although PD is now known to be a multisystem neurodegenerative disorder, most therapeutics are only aimed at alleviating the motor symptoms. Thus far, the most successful breakthrough in the symptomatic treatment of PD is levodopa (L-DOPA), a precursor to dopamine. L-DOPA was originally shown to dramatically alleviate the slowness of movement, increased muscle tone and tremor that are typical of PD [11]. Even so, L-DOPA has never been shown to be effective against any non-motor symptoms or even slow the degeneration of DA neurons in the substantia nigra. Thus treatment with L-DOPA works optimally early on, but over time can lead to dyskinesias [12]. Because of this, there is a desperate need for treatments that can significantly alter the progression of PD which demands a more in depth understanding of the mechanisms underlying disease progression.

A striking feature of DA neurons is their high energetic demands, which require them to sustain a high level of mitochondrial activity. This increase in mitochondrial activity comes at a cost to the neuron in the form of superoxide and reactive oxygen species that can be damaging to the mitochondria and toxic to the neuron [13]. Thus mitochondrial dysfunction is often speculated to play a major role in the development of PD, an idea supported by the association of mitochondrial toxins such as MPTP, rotenone, and paraquat with sporadic PD [14].In addition, many of the genes associated with PD also implicate mitochondria in disease pathogenesis. It has been found that six genes can be conclusively linked to PD: Park1 or α-synuclein, Park2 or Parkin, Park5 or UCH-L1, Park6 or PINK-1, Park7 or DJ-1, and Park8 or LRRK2 [15].

Although mitochondrial dysfunction has long been associated with PD, only recently has it been seriously considered to play a major role in disease progression. Several mechanisms have been investigated for how exactly the mitochondria are involved in neuronal death. The most prominent theory is that complex I deficiency leads to an increase in reactive oxygen species which then cause damage and toxicity to neighboring mitochondria. This is supported by data showing that complex I is the main site of reactive oxygen species production in the mitochondria because of its responsibility to transfer a single electron to oxygen as part of the respiratory chain [16]. In conjunction with this theory, researchers have speculated that the possibly disease causing alterations in complex I activity might actually be the result of mutation in the mitochondrial DNA. This idea is supported by the established age-dependent increase in mitochondrial DNA deletions that happens in the substantia nigra, leading to respiratory chain dysfunction [17]. A less prominent hypothesis for the underlying mechanism of oxidative stress is that of calcium signaling. Calcium channels are known to play an essential role in the constitutively active action potentials unique to dopaminergic neurons. It has been shown that

maintained opening of these channels causes oxidative stress and toxicity, thus implicating that disruption of calcium homeostasis might contribute to PD pathogenesis [18].

Another possibility to describe the link between mitochondria and PD is that a defect in the elimination or turnover of mitochondria can lead to respiratory dysfunction over time or generate increased levels of damaging reactive oxygen species. Maintenance of the mitochondrial network is achieved through a complex balance of fission and fusion and is not fully understood. This is because although cells can destroy damaged mitochondria, they cannot create them and thus rely on fission to maintain the number of mitochondria constant [19]. In fact it has even been shown that the processes are interconnected and dependent in such a way that fission followed by selective fusion can signal mitochondrial degradation [20]. Two genes that have been shown to be important factors in maintaining mitochondrial integrity and that are also genes identified in hereditary PD are PINK1 and parkin. Defects in these proteins could be responsible for an impairment of mitochondrial turnover thus leading to oxidative stress and eventually neuronal cell death. In particular, mutations in parkin are the most frequent cause of juvenile PD [21].

Parkin is an E3 ubiquitin ligase that is able to catalyze a variety of ubiquitination reactions associated with a number of cellular responses. The best studied role of parkin is its interaction with PINK1 to orchestrate the degradation of damaged mitochondria by autophagy, a process termed mitophagy. Once a mitochondrion becomes depolarized by oxidative damage, PINK1 is stabilized on the outer mitochondrial membrane in order to recruit parkin [22]. By ubiquitinating several proteins on the outer membrane, parkin can then signal for the mitochondrion to be degraded [23]. In addition, parkin has been implicated in other processes like resistance to intracellular pathogens [24] and even in certain cancers. Interestingly, an increasing body of evidence has shown that parkin inactivation increases the likelihood of developing cancer and

that parkin overexpression inhibits the migration and invasion of multiple cancer cells [25]. Specifically, parkin was found to bind to α/β tubulin and cause their degradation via ubiquitination, which could provide insight into its presumed anti-cancer role [26].

More recently, parkin's possible role in cell proliferation has been further evaluated. Cell proliferation is a fundamental biological process that operates through the most complex interplay of signals in order to drive organism development and maintain adult tissue homeostasis. The driving factor for progression through the cell cycle, from G1 to S to G2 is the oscillation of two classes of protein complexes, the cyclin-dependent kinases and the E3 ubiquitin ligases. Two specific types of cyclin-dependent kinase, the cyclin-D and cyclin-E dependent kinases play major roles in the G1/S transition and are commonly deregulated in cancer. Interestingly, parkin was found to coordinate the activities of both the D and E cyclin-dependent kinases during the cell cycle [27]. This suggests that parkin might have effects in the cell that have not yet been studied. Parkin was even found in a genome-wide analysis as a candidate gene for attention-deficit/hyperactivity disorder [28].

The interaction of parkin with tubulin was verified in a recent high throughput screen we performed to find proteins that interact with microtubules. An unexpected result of this screen was the interaction between parkin and an iron shuttling protein, lactoferrin. Lactoferrin is a member of the transferrin family of proteins. It is a non-heme iron binding glycoprotein with a molecular weight of 80 kDa. Although it is a major component of bovine and human milk, it is also one of the major proteins in all exocrine secretions including saliva, tears, and mucus. It is also known for its anti-bacterial, anti-fungal, anti-viral, anti-oxidant, anti-inflammatory, anti-parasitic, anti-allergic, and anti-cancerous properties [29]. Originally it was thought that lactoferrin's primary role was as an iron transport protein in the blood or as a general iron

chelator, but the discovery of lactoferrin in neutrophils suggests that it might have a greater role in innate immunity [30]. It was found to be stored in specific granules of neutrophilic leukocytes until signaled to be released during an inflammatory response [31].

As a key component in inflammatory responses it is no surprise that lactoferrin was also found to be upregulated in activated microglia in the brain [32]. In addition, iron's role in the central nervous system has been established for decades. It is known that iron is involved in the synthesis of many neurotransmitters including dopamine and serotonin [33]. In order to successfully carry out its function, iron must be concentrated into pools rather than be evenly distributed. The basal ganglia in particular, were shown to maintain impressively high iron concentrations [34]. Excessively high concentration of free iron, however, can cause oxidative stress by reacting with hydrogen peroxide. Lactoferrin then, could play a part in transporting iron in the brain to maintain the delicate iron balance. Although not typically associated with neurodegenerative diseases, this could suggest that lactoferrin is actually a major component in disease progression. In fact, lactoferrin was even found within the neuropathologic lesions of Alzheimer's disease [35]. In Parkinson's disease cases it was found that the neurodegerative process affects lactoferrin-stained neurons because less were seen in the substantia nigra of diseased tissue. The levels of lactoferrin, however, were increased in the neurons that were left in the substantia nigra and ventral tangential regions compared to control cases [36].

In the current study I examined the novel interaction between parkin and lactoferrin. Immunoprecipitation experiments confirmed the interaction between parkin and lactoferrin. This prompted me to analyze lactoferrin and iron levels in the brains of parkin knockout mice. No significant changes in lactoferrin levels were seen in general by western analysis or specifically in the subtantia nigra and adjacent regions by immunofluorescent staining. In addition the levels

of iron in the brain were not significantly altered in the knockout mice. In summary, although parkin and lactoferrin may interact in the mouse brain, the presence or absence of parkin did not affect lactoferrin or iron levels.

Materials and Methods:

Mice

Animal care was conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at the University of California in Los Angeles. Parkin KO mice were generated as previously described [37]. Genotypes were determined and confirmed by polymerase chain reaction.

Brain sample preparation for Co-Immunoprecipitation and Western blotting

Samples were homogenized in tissue weight (mg) multiplied by 8 microliters of cold modified RIPA buffer. This homogenate was left on ice for ten minutes and centrifuged at 13,000g at 4 degrees C for 15 minutes. Supernatant was then collected and protein concentration was measured. 2x sample buffer was added in equal volume to protein lysate and sample was boiled at 100 degrees C for ten minutes. Modified RIPA buffer contained 50mM Tris-HCl, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1% NP-40, 1 tablet of 25x protease inhibitor cocktail, pH 7.4.

Co-Immunoprecipitation

40 ul of Magnetic Protein A and protein G beads were used. First they were washed three times in modified RIPA buffer. An equal volume of lysate and antibody was added to the beads. The

reaction was left to bind at 4 degrees for 3-4 hours on a rotator. The reactions were washed two times in lysis buffer and sample buffer was added. The sample was boiled for ten minutes and centrifuged. Supernatant was collected and stored at -20 degrees C.

Western Blot

Equal volume of sample was run on pre-made gradient gels then transferred onto PVDF membranes, blocked, left in primary antibody overnight at 4 degrees C, washed, incubated in secondary antibody at room temperature for 30 minutes and scanned on a LICOR scanner. Primaries used were mouse anti-lactoferrin (Abcam, 1:1000), rabbit anti-Parkin (Cell Signaling or Abcam, 1:1000), rabbit anti-GAPDH (Abcam, 1:2000).

Immunofluorescence

Mice were deeply anesthetized with pentobarbital (100mg/kg i.p.) and intracardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were rapidly removed, postfixed in 4% PFA overnight, then cryoprotected in 30% sucrose in 0.1M PBS until they sank to the bottom of the tube. They were then frozen in dry ice and stored at -80 degrees C. Tissue sections from the substantia nigra were collected using a cryostat (40um thickness). These sections were then stained for lactoferrin via the following protocol: sections were washed in 0.05M tris-buffered saline (TBS) three times for ten minutes each, blocked in mouse on mouse blocking reagent (vector lab, PK-2200) for one hour, washed in TBS for five minutes, incubated in 10%NGS/0.2% Trition X for one hour, incubated in mouse anti-lactoferrin (Abcam, 1:500) overnight, washed in TBS three times for ten minutes each, incubated in Alexa Fluor 688 goat anti-mouse for two hours, and finally washed in TBS three times for ten minutes each.

Perl's Staining

Perl's staining is used to detect the presence of iron. The stain uses solutions of potassium ferrocyanide and acid to stain tissue. Using substantia nigra sections prepared as above, the following protocol was used: wash in 0.1M PBS for ten minutes, wash in ddH2O for 30 seconds, incubate in 2% HCl and 2% Potassium Ferrocyanide for 30 minutes, wash in 50mM TBS for one minute, incubate in 0.5%H2O2 in 50mM TBS for 15 minutes, and finally leave in DAB solution for 30 minutes to observe the reaction.

Results

Parkin binds lactoferrin in mouse brains

To confirm that parkin indeed interacts with lactoferrin in mice we performed a co-immunoprecipitation in wild-type mouse brain extract using both a parkin and lactoferrin antibody (fig1). Immunoglobulin was used as a control and as expected did not immunoprecipitate any protein (IgG lane). Where the parkin antibody was used, both parkin and lactoferrin appeared on the blot and where the lactoferrin antibody was used, again both lactoferrin and parkin appeared on the blot. This indicates that they interact in mouse brains.

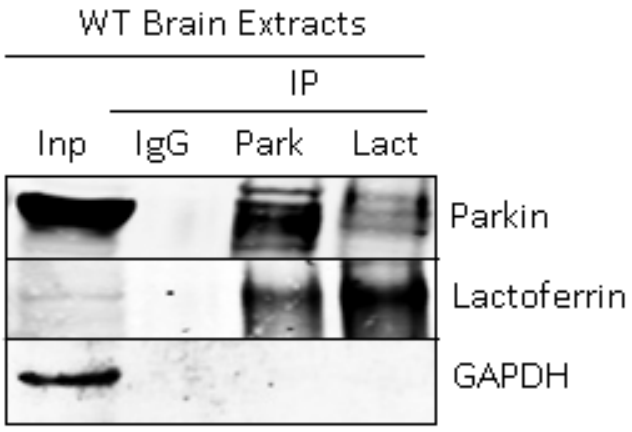


Fig 1. **Parkin binds lactoferrin in mouse brains.** Co-Immunoprecipitation followed by western analysis of right hemisphere brain homogenate from 7 month old wild-type mouse confirms interaction.

Analysis of parkin and lactoferrin levels in mouse brain extracts

Because we previously found in cells that parkin ubiquitinates lactoferrin, I decided to examine the effects of parkin knockout on lactoferrin levels in seven or two month old wild-type, heterozygous, and knockout mouse brain extracts (fig 2). Lactoferrin levels remained unchanged even in knockout mice. The second row which was stained with a parkin antibody as well as PCR analysis confirms knockout. Interestingly, in the third row, the other parkin antibody that I used stained even the knockout extracts at the same size as the one in the second row.

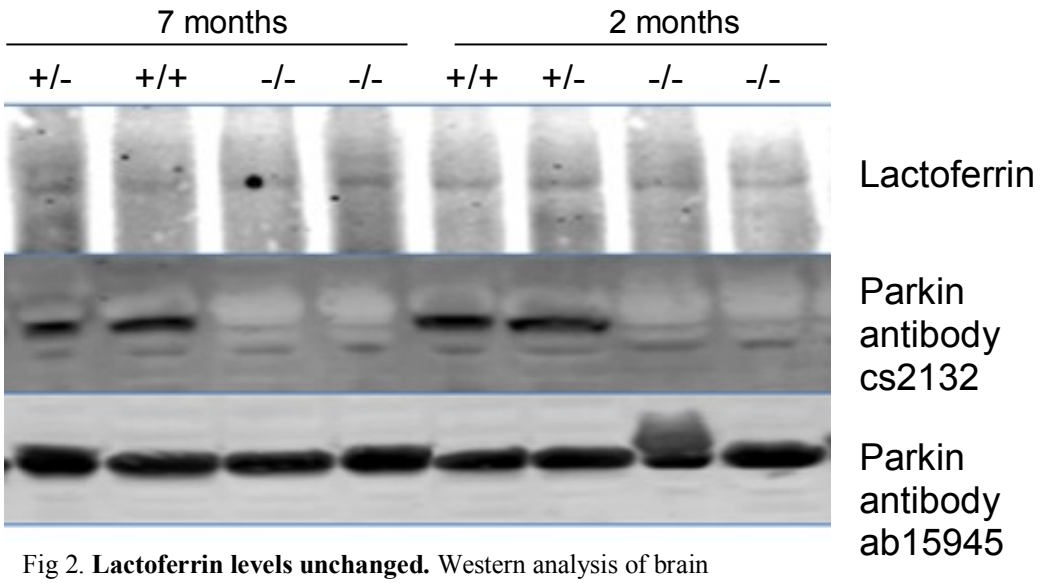


Fig 2. **Lactoferrin levels unchanged.** Western analysis of brain homogenate from 7 and 2 month old wild-type, heterozygous, and knockout mice stained with lactoferrin and two different parkin antibodies.

Analysis of cytoplasmic lactoferrin levels by immunofluorescence

To gain insight into the exact cellular levels of lactoferrin within the substantia nigra and adjacent regions, we stained substantia nigra containing sections with the lactoferrin antibody and alexa fluor 688 then analyzed the sections using confocal imaging (fig 3). Lactoferrin fluorescene intensity was quantified in seven wild-type and nine knockout sections from mice 3-4 months of age. No differences in lactoferrin levels were found; indicating that parkin knockout in mice does not affect overall lactoferrin levels.

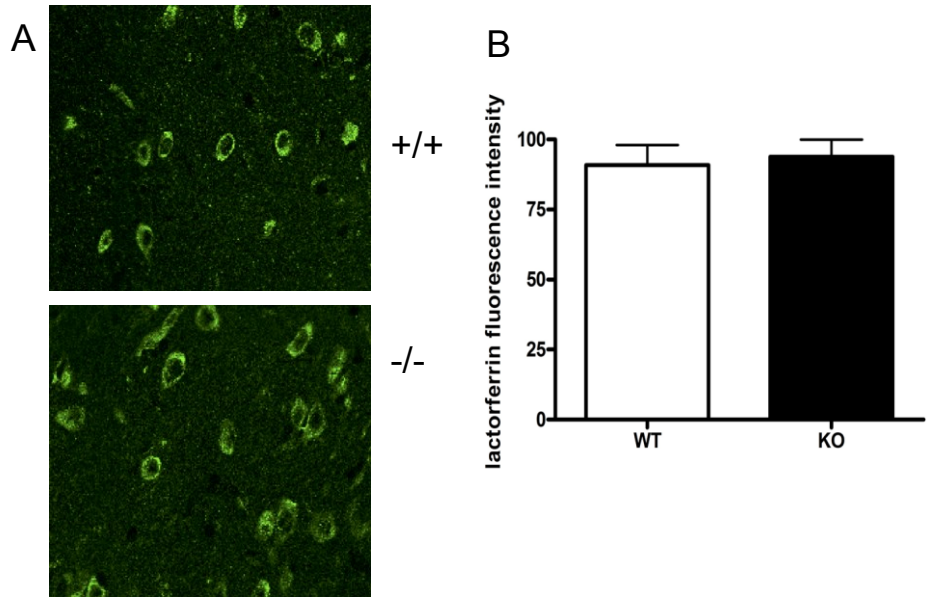


Fig 3. **Cytoplasmic lactoferrin levels unchanged.** A. Representative confocal images of immunofluorescently stained brain sections from wild-type and knockout mice. B. Cytoplasmic lactoferrin was quantified using imageJ and t-test was performed, wild-type n=7, knockout n=9, 3-4 months of age.

Analysis of total iron levels

Even if lactoferrin levels were not altered by parkin knockout, there still may be an effect on lactoferrin activity. To try and gain insight into lactoferrin activity we performed Perl's staining for total iron levels on sections from the same group of mice used in the immunofluorescence experiments (fig 4).

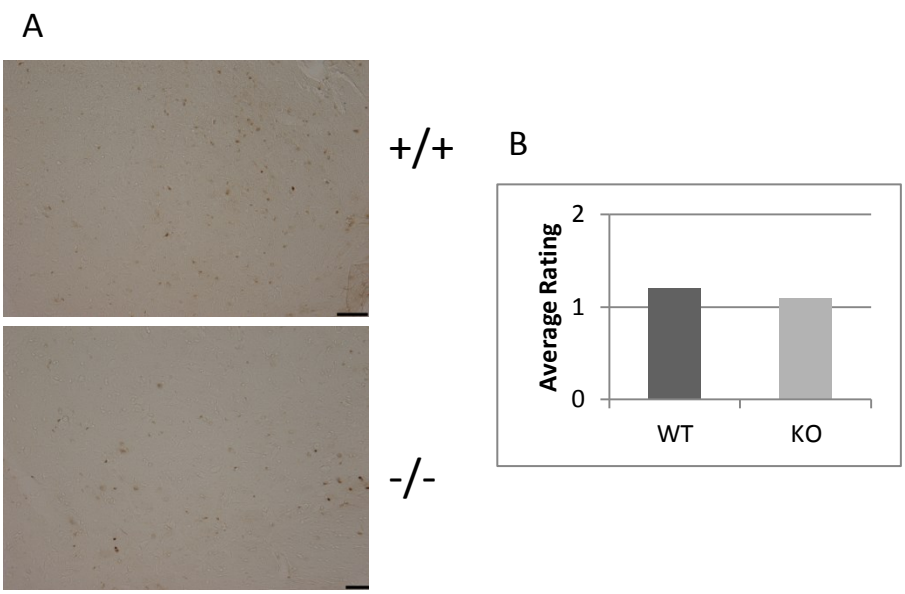


Fig 4. **Iron levels unchanged.** A. Representative images of Perl's stained brain sections from wild-type and knockout mice, 3-4 months of age, scale bar 50um. B. Iron staining intensity was manually rated by blinded researcher. Each section was assigned a number of intensity of 0, 0.5, 1, 1.5, or 2 and average was taken. Wild-type n=10, knockout n=9.

Discussion

Preliminary data suggested that the E3 ubiquitin ligase parkin and the iron binding protein lactoferrin may interact in vivo. The discovery of this interaction is of profound importance and offers significant implications for furthering our understanding of PD. There are many links currently associated with the development of PD; yet uncovering the network in which they connect in such a way to cause the selective degeneration of dopaminergic neurons has been a monumental challenge. A binding interaction between parkin and lactoferrin can help bridge that gap by implying a relationship between oxidative stress and iron metabolism. Although they have been confirmed to interact, the effect of their binding and thus the details of their association remain elusive and were not fully uncovered in the present study.

The aim of this study was to test this hypothesis in parkin knockout mice to bring to light the consequences of this binding and its possible implications in PD. Initial co-immunoprecipitation experiments confirmed the interaction occurs in mouse brains and thus allowed us to further investigate its meaning. Western blot analysis, however, did not support the hypothesis that lack of parkin leads to an accumulation of lactoferrin in the brain. Lactoferrin levels were constant in all the samples, suggesting that if parkin does indeed ubiquitinate lactoferrin it may not target it for destruction, but could still change its localization pattern.

In addition, there was a surprising inconsistency between antibodies. The genotypes which were initially determined and confirmed by PCR analysis, show that there is knockout of parkin, as does one of the antibodies. The other, however, shows a parkin band in all of the mice. This could mean that in mice there is another parkin isoform or parkin-like protein recognized by that antibody. Understanding why this inconsistency is present warrants further experimentation and might explain the lack of deficits seen in these mice. The presence of additional parkin-like proteins specific to mice could also explain their accepted higher tolerance to neuronal degeneration.

To further analyze lactoferrin levels in a region-specific way, substantia nigra containing sections were stained immunofluorescently. Again, no changes in lactoferrin levels or distribution were seen. This further supports the notion that parkin knockout does not affect lactoferrin levels in areas near the substantia nigra in mouse brains. This could mean that ubiquitination of parkin by lactoferrin is not a signal for destruction or for delocalization. It could be that lactoferrin instead becomes inactivated or stabilized by the ubiquitination event.

Lastly, iron levels in substantia nigra containing sections were evaluated. No changes in intensity were seen between the wild-type and knockout sections implying that parkin knockout does not cause iron to accumulate. This suggests that lactoferrin activity has either not been altered, or is being compensated for. Iron levels are tightly regulated in the brain and it might also be that a difference in iron levels isn't apparent until later time points. Another theory is that although total iron remains the same, deactivation of lactoferrin causes an increase in free iron that can react with hydrogen peroxide and cause oxidative stress. This oxidative stress can then lead to mitochondrial dysfunction which is associated with development of PD.

It has been shown previously that a deficiency in parkin alone in mice isn't enough to cause dopaminergic neuron degeneration or other PD associated deficits [37]. As a result it has been postulated that parkin might actually be neuroprotective, especially since its overexpression has been shown to have beneficial effects [38]. If this were the case, interaction with lactoferrin could illuminate the mechanism of action leading to the neuroprotective effects. The ubiquitination of lactoferrin could act as a signal for other types of modification that alter or improve its function. As a result, total levels remain the same, but in the presence of parkin the brain is more effectively protected from potential future damage.

In general, further experimentation is needed to elucidate the effects of parkin on lactoferrin and iron metabolism. More time points and brain regions could be included to ensure there isn't a region specific effect. An antibody for ubiquitinated lactoferrin could be used to look for an increase in the modified form. In addition, parkin mutant models could be examined. Similar measurements can be made in other PD mouse models to relate these proteins to the other well-known factors associated with the disease. Further mitochondrial studies can be done to assess the effect of parkin knockout on mitophagy and its possible link with a conversion from non-

toxic to toxic iron. This study demonstrated that although there is interaction between parkin and lactoferrin, the effects are likely part of a more complex pathway in mice.

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