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Parkin prevents cortical atrophy and A β -induced alterations of brain metabolism: ^{13}C NMR and MRI studies in AD models

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Abstract

Alzheimer's disease (AD) is a neurodegenerative aging disorder characterized by extracellular A β plaques and intraneuronal neurofibrillary tangles. We conducted longitudinal studies to examine the effects of A β on brain amino acid metabolism in lentiviral A β_{1-42} gene transfer animals and transgenic AD mice. We also performed lentiviral parkin gene delivery to determine the effects of A β clearance in AD models. A β_{1-42} activated mTOR signaling, and increased 4E-BP phosphorylation. A β_{1-42} increased the synthesis of glutamate and aspartate, but not glutamine, leucine and isoleucine, but an increase in leucine and isoleucine levels was concurrent with diminution of neurotransmitters. Additionally, A β_{1-42} attenuated mitochondrial TCA cycle activity and decreased synthesis of its by-products. Glutamate levels increased prior to lactate accumulation, suggesting oxidative stress. Importantly, parkin reversed the effects of A β_{1-42} on amino acid levels, prevented TCA cycle impairment and protected against glutamate toxicity. Cortical atrophy was observed in aged 3xTg-AD mice, while parkin expression was associated with reduced atrophy. Similarly, A β_{1-42} resulted in significant cell loss, pronounced astrogliosis and cortical atrophy and parkin reduced astrogliosis and reversed A β_{1-42} effects on cell loss and cortical atrophy. Taken together these data suggest that parkin prevents amyloid-induced alteration of brain metabolism and may be used as a therapeutic target to limit neuronal loss in AD.

Keywords

Amyloid; brain metabolism; amino acids; mitochondria; atrophy; parkin

Introduction

Alzheimer's disease (AD) pathology is characterized by deposition of intraneuronal hyperphosphorylated Tau and extracellular β -amyloid (A β plaques (Hardy and Selkoe, 2002). Cleavage of amyloid precursor protein (APP) by the β -site APP cleaving enzyme (BACE1)

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near the C-terminus results in the formation of APP C-terminal fragment (CTF β) C99. Subsequent cleavage of CTF β by γ -secretase generates A β ₄₀ or A β ₄₂ (Jarrett et al., 1993), which are produced intracellularly (Cook et al., 1997, Xu et al., 1997, Lee et al., 1998, Skovronsky et al., 1998, Greenfield et al., 1999) and deposited in amyloid plaques in AD brains (Wilson et al., 1999, Gouras et al., 2000). Accumulation of intracellular A β contributes to deposition of extracellular plaques (Gouras et al., 2000, D'Andrea et al., 2001). Intracellular A β plays an important role in AD pathogenesis (Cataldo et al., 1997) as accumulation of intraneuronal A β precedes the onset of cognitive symptoms (Cataldo et al., 2000). Parkin is a ubiquitin E3 ligase involved in proteasomal degradation of misfolded proteins (Shimura et al., 2000). Mutations in Park-2 gene result in loss of parkin function and are associated with autosomal-recessive juvenile onset Parkinson's Disease (ARJPD) (Kitada et al., 1998, Lucking et al., 2000). The most reproducible function of parkin is its pan-protective activity in proteasomal degradation and defense against mitochondrial insults and oxidative stress (Shimura et al., 2000). Parkin ubiquitinates intracellular A β ₁₋₄₂ and stimulates its removal via the proteasome or the autophagy-lysosome system (Burns et al., 2009, Rosen et al., 2010, Khandelwal et al., 2011b).

Kinase signaling via the mammalian target of rapamycin (mTOR) regulates mRNA translation through phosphorylation of downstream kinases such as eukaryotic initiation factor 4E (eIF-4E) binding protein (4E-BP) and p70S^{6k} (Shima et al., 1998, Gingras et al., 2001, Grolleau et al., 2002, Mamane et al., 2004). mTOR promotes the phosphorylation and activation of p70S^{6k} and the hierarchical phosphorylation of 4E-BPs (Gingras et al., 2001, Jacinto and Hall, 2003, Kim et al., 2003, Ruggero and Pandolfi, 2003, Kim and Sabatini, 2004). 4E-BP interacts with its downstream effector eIF-4E (Marcotrigiano et al., 1999). Hypo-phosphorylated 4E-BP strongly binds to eIF-4E, whereas hyper-phosphorylation leads to 4E-BP release from eIF-4E and subsequent activation of cap dependent translation (Gingras et al., 1999, Gingras et al., 2001). 4E-BP is phosphorylated at multiple sites in a hierarchical manner (first Thr37 and Thr46, then Thr70 and last Ser65) (Gingras et al., 1999, Gingras et al., 2001). Rapamycin treatment inhibits 4E-BP phosphorylation, increases the interaction between eIF-4E and 4E-BP, and consequently inhibits cap-dependent translation (Gingras et al., 2001, Grolleau et al., 2002). eIF-4E is phosphorylated at Ser209 by MAP kinase signaling (Fukunaga and Hunter, 1997, Waskiewicz et al., 1997).

To determine the effects of parkin on metabolic and anatomical brain changes in expressing animals, we expressed parkin and conducted longitudinal high frequency ¹³C NMR and MRI studies in triple transgenic AD (3xTg-AD) mice, which over-express APP(Swe), Tau(P301L) and harbor the PS1(M146V) knock-in mutation and gene transfer rat models expressing lentiviral A β ₁₋₄₂. The 3xTg-AD mice show little cell death and progressive accumulation of A β and Tau (Oddo et al., 2003a, Billings et al., 2005, Oddo et al., 2006), whereas the lentiviral A β ₁₋₄₂ model displays faster degeneration and Tau accumulation (Rebeck et al., 2010), enabling us to compare the early versus progressive effects of A β on brain metabolism and morphometric changes. These studies provide an insight into the mechanisms of parkin-mediated restoration of brain metabolism and prevention of atrophy in AD models.

Experimental procedures

Stereotaxic surgery was performed to inject 1×10^{12} m.o.i lentiviral parkin into the right, and 1×10^{12} m.o.i lentiviral LacZ into the left CA1 region of the hippocampus of 1-1.5 year old 3xTg-AD mice (Khandelwal et al., 2011b). We also injected 1×10^{12} m.o.i lentiviral A β ₁₋₄₂+ 1×10^{12} m.o.i lentiviral LacZ into the left M1 primary motor cortex of 2-month old Sprague Dawley rats, and 1×10^{12} m.o.i lentiviral β ₁₋₄₂+ 1×10^{12} m.o.i lentiviral parkin into the right hemisphere as previously described (Burns et al., 2009). A total of 84 mice were

injected and sacrificed 3 months post-injection and 86 rats were injected and sacrificed 2–12 weeks post-injection. All animal surgery and handling was approved by Georgetown University Animal Care and Use Committee (GUACUC).

Mammalian Target of Rapamycin (mTOR) activity assay

To assess the activity of mTOR in cortical brain lysates, we used a colorimetric Enzyme-linked immunosorbent assay (ELISA)-based activity, utilizing a p70S6K GST fusion protein as a specific mTOR substrate. mTOR was immuno-precipitated using a rabbit polyclonal antibody (1:100) for total mTOR (Cell Signaling Inc) and the kinase activity was measured in immunoprecipitated brain lysates according to manufacturer's protocol (EMD Millipore, Cat # CBA055).

Western blot analysis—The cortex was dissected out and homogenized in 1xSTEN buffer then centrifuged at 5.000g and the supernatant was collected. Rabbit polyclonal antibodies for total 4E-BP1 (1:1000), Thr 37/46 phosphorylated 4E-BP1 (1:500), Ser 209 phosphorylated eIF-4E (1:500) and Thr 389 phospho-p70S^{6K} (1:500) were used (Translational control sampler kit, Cell Signaling Technology). Rabbit polyclonal antibodies for total mTOR (1:1000) and Ser 2448 phosphorylated mTOR (1:1000) were used (Translational control sampler kit, Cell Signaling Technology). Tumor necrosis factor- β (TNF- α) was probed with (1:1000) anti-TNF- α rabbit antibody (Serotec), inducible nitric oxide synthase (iNOS) was probed with (1:1000) anti-iNOS rabbit antibody (BD Transduction Laboratories, USA). Western blots were quantified by densitometry using Quantity One 4.6.3 software (Bio Rad). Densitometry was obtained as arbitrary numbers measuring band intensity. Data were analyzed as mean $\pm$ Standard deviation, using ANOVA, with Mann Whitney comparison between treatment groups.

Immunohistochemistry

Animals were deeply anesthetized with a mixture of Xylazine and Ketamine (1:8), washed with 1X saline for 1 min and then perfused with 4% paraformaldehyde (PFA) for 15–20 min. Brains were quickly dissected out and immediately stored in 4% PFA for 24h at 4°C, and then transferred to 30% sucrose at 4°C for 48h. Immunohistochemistry was performed on 20 micron-thick sections. Astrocytes were probed (1:200) with monoclonal anti-glial fibrillary acid protein (GFAP) antibody (Millipore Corporation, USA). Cresyl violet (Nissl) staining was performed according to manufacturer's instructions (Sigma).

Stereological methods were applied by a blinded investigator using stereological counting (Stereologer, Systems Planning and Analysis, Chester, MD) of cresyl violet or GFAP stained cells. To determine the total positive cell counts in the rat, 20 cortical fields were assessed from at least 10 brain sections (~400 positive cells per animal) from each animal. In the rat, stereological counting was performed within the area of viral distribution across 2.5 mm radius from the point of injection within the cortex. All values were averaged to account for the gradient of staining across 2.5 mm radius and compared to control or other treatments. An optical fractionator sampling method was used to estimate the total number of positive cells with multi-level sampling design. Cells were counted within the sampling frame determined optically by the fractionator and cells that fell within the counting frame were counted as the nuclei came into view while focusing through the z-axis.

High frequency ¹³C/¹H Nuclear Magnetic Resonance (NMR) Spectroscopy—

Animals were fasted overnight with free access to tap water and were intra-peritoneally (I.P) injected with [1-¹³C] glucose solution (0.5 mol/L) over 10s (0.3 ml/25–30 g body weight; 200 mg/kg). 45 min later, animals were sacrificed by cervical dislocation and hemispheres were isolated and immediately homogenized in 6% ice-cold perchloric acid, 50 mM

NaH₂PO₄. After homogenization and lyophilization, extracts were re-suspended in 0.65 ml D₂O containing 2 mM sodium [¹³C]formate as internal intensity and chemical shift reference (δ 171.8). Metabolite pool size was identified on ¹H {¹³C-decoupled} NMR spectra. Peak areas were adjusted for nuclear Overhauser effect, saturation and natural abundance effects and quantified by reference to [¹³C] formate. Metabolite pool sizes were determined by integration of resonances in fully relaxed 400 MHz {¹³C-decoupled} ¹H spectra using N-acetylaspartate as internal intensity reference. Incorporation of ¹³C into isotopomers was measured in reference to [¹³C]formate. All data were collected on a 9.7 Tesla Varian Spectrometer with dual ¹³C/¹H probe. {¹³C-decoupled}-¹H spectra were acquired with 3000 scans, pulse width 45°, relaxation delay 1s, line broadening 0.5 Hz, acquired data points 13.132 and transformation size 32K at room temperature. {¹H-decoupled}-¹³C spectra were acquired with 24000 scans and 31875 data points. Spectra were integrated and quantified using MestReNova software (Master Lab Research).

Brain MRI *in vivo* was performed on a 7-T Bruker BioSpec 20cm horizontal bore (Bruker Biospin, Bellerica, MA). All animals were placed in a probe with a stereotaxic holder to ensure that the MRI scans are perfectly perpendicular to the animal brain. To obtain MRI scans of the brain, the ¹H frequency (300 Mega-Hertz) was used to find the center frequency and the magnetic field was linearized (shimmed) over the whole animal brain. ¹H scout images are obtained in transverse, coronal, sagittal and 3D magnetic resonance images were acquired. The MRI template set was created from multi-slice T₂-weighted images of the rat brain acquired using a Rapid Acquisition with Refocused Echoes (RARE) sequence with the following acquisition parameters: matrix 256 × 256; Field-of-view (FOV) 40 mm³, slice thickness 1 mm, 16 contiguous coronal slices; with Echo Time (TE) = 11-milliseconds; Repetition Time = 7 seconds; Receiver Band width 7 kilohertz, Points acquired were 1024. A 72-mm birdcage resonator was used for RF transmit and the Bruker quadrature 'RatBrain' coil was used for RF receive. The images were acquired in the coronal plane, centered 10 mm caudal from the posterior edge of the olfactory bulb. The parameters used in the mouse brain scans were T₂-weighted, RARE sequence, FOV = 12 × 12 × 24 mm and matrix size = 200 × 200, slice thickness 300 m, 10 contiguous coronal slices; with Echo Time (TE) = 11-milliseconds; Repetition Time = 7 seconds Receiver Band width 7 kilohertz. To perform MRS in mouse brains, a voxel size was set to 7 mm × 5.5 mm × 10 mm. Shimming was performed on this volume element until the full-width half maximum H₂O line width is 5 Hz or smaller. MRS was conducted just prior to the imaging experiment. At the onset of the experiment the ¹H frequency is used to find the center frequency and the magnetic field is shimmed over the brain region of the animal. The PRESS (Point-RESolved Spectroscopy) sequence used had echo time (TE) = 13.77 ms, repetition time (TR) = 1.5 s, spectral width = 3472.22 Hz, number of points acquired were 1024, number of averages (NA) = 512, gradient spoiling time = 0.5 ms, excitation pulse length 10 μs, refocusing pulse length 1000 ms. Spectra were obtained after localizing the voxel 2×2×2 mm³ on cortical brain region, the field is shimmed and spectra were acquired. Spectra were processed with 6 Hz line broadening. Automatic baseline adjustment was performed using XWINMR Version 6.2 software (Bruker BioSpin, Bellerica, MA), the NAA/NAAG peak was calibrated to 1 and set as an internal reference, and all other peaks were integrated relative to NAA/NAAG. Volumetric evaluation and segmentation of brain images were performed using NIH Image J and Insight ITK-SNAP computer software, which implement 3D active contour segmentation methods. Spherical bubbles were placed inside the ventricles as starting points for the snake evolution, and the software was allowed to run 50–60 iterations until the bubbles fully fill the ventricles or the intracranial space, which is defined as the area between the skull and the brain. The software recorded data in cm³ and the values were converted into percentage control.

Results

Lentiviral A β ₁₋₄₂ activates mTOR and 4E-BP signaling

We previously showed lentiviral expression of A β ₁₋₄₂ in gene transfer animal models and demonstrated its effects on cell death, autophagy and inflammation (Burns et al., 2009, Rebeck et al., 2010). In the present studies we examined the effects of lentiviral A β ₁₋₄₂ on markers of the mTOR signaling pathway. ELISA measurements showed that lentiviral expression of A β ₁₋₄₂ in the rat cortex significantly increased (61%, N=8) the activity of mTOR compared to LacZ injected brains four weeks post-injection (Fig. 1A). We pursued another approach to examine the effects of A β ₁₋₄₂ on mTOR signaling using Western blot analysis. Lentiviral A β ₁₋₄₂ significantly increased (41%, by densitometry, N=8) total mTOR levels (Fig. 1B, 1st blot) and phosphorylation at Ser 2448 (Fig. 1B, 54%, 2nd blot) compared to LacZ injected motor cortex. To confirm the effects of A β ₁₋₄₂, we determined the phosphorylation of p70S^{6k}. No changes in the levels of total p70S^{6k} were observed (Fig. 1B, 3rd blot), but lentiviral A β ₁₋₄₂ significantly increased (36%, by densitometry) the p70S^{6k} phosphorylation at Thr389 (Fig. 1B 4th blot) relative to actin. Further examination of kinase levels revealed no significant changes in total 4E-BP (Fig. 1C, 1st blot) relative to actin (4th blot). However, we observed a significant increase in the levels of Thr 37/46 phosphorylated (38%, N=8) 4E-BP1 (Fig. 1C, 2nd blot), indicating increased kinase activity in A β ₁₋₄₂ over-expressing animals. A significant decrease (65%, by densitometry, N=8) in the phosphorylation level of eIF-4E at Ser 209 was observed downstream of 4E-BP1, suggesting that A β ₁₋₄₂ increases 4E-BP1 phosphorylation and decreases eIF-4E phosphorylation.

A β ₁₋₄₂ increases amino acid levels and parkin reverses these effects

Because we observed alterations in translational control in the presence of lentiviral A β ₁₋₄₂ four weeks post-injection, we examined the levels of amino acids including leucine, isoleucine, glutamate, glutamine and aspartate. We co-injected lentiviral parkin and A β ₁₋₄₂ (Khandelwal et al., 2011b) and performed longitudinal studies to determine metabolic changes in gene transfer animal models. We performed high frequency ¹³C/¹H NMR spectroscopy in brain extracts to compare A β ₁₋₄₂, LacZ, parkin and parkin+A β ₁₋₄₂ injected hemispheres at 2 and 4 weeks post-injection. No differences in the flux of ¹³C label into leucine C4 and isoleucine C4 (Fig. 2A), and no changes in amino acid pool sizes (Fig. 2B) were observed between A β ₁₋₄₂ and LacZ injected cortex two weeks post-injection. However, flux into leucine C4 and isoleucine C4 (Fig. 2A), and amino acid pool sizes (Fig. 2B) were significantly increased (N=5) between A β ₁₋₄₂ and LacZ injected brains four weeks post-injection. We previously showed that parkin alone did not change brain metabolism compared to LacZ (Khandelwal et al., 2011b), but co-injection of lentiviral parkin+A β ₁₋₄₂ reversed the changes in leucine and isoleucine levels back to control four weeks post-injection (Fig. 2A&B).

The flux of ¹³C label into glutamate C4 was significantly increased (P<0.05, N=5) two weeks but decreased four weeks post-injection with A β ₁₋₄₂ compared to LacZ (Fig. 2C), however, parkin co-expression with A β ₁₋₄₂ reversed the changes in C4 glutamate levels (Fig. 2C). The total level of glutamate was also significantly increased at two weeks, and decreased four weeks post-injection (Fig. 2D), in agreement with metabolic flux. Parkin expression prevented changes in glutamate levels four weeks post-injection (Fig. 2D), consistent with our previous findings in 3xTg-AD mice (Khandelwal et al., 2011b). No significant differences were observed in metabolic flux into glutamine C4 (Fig. 2C) or total glutamine levels (Fig. 2D) two weeks post-injection, suggesting that the increase in glutamate levels are independent of the glutamate-glutamine cycle. ¹³C flux into glutamine C4 was significantly decreased four weeks post-injection with A β ₁₋₄₂ (Fig. 2C), along with the total level of glutamine (Fig. 2D) in A β ₁₋₄₂ compared to LacZ injected animals. Parkin

reversed glutamine decrease back to control (Fig. 2C&D), suggesting that A β ₁₋₄₂ increases glutamate levels two weeks post-injection but at four weeks both glutamate and glutamine levels are decreased.

We detected a significant increase in ¹³C flux into aspartate C3 (Fig. 2E) two weeks post-injection with A β ₁₋₄₂, but flux into both aspartate C2 and C3 was significantly decreased four weeks post-injection (Fig. 2E) compared to LacZ. Total aspartate levels were increased two weeks and decreased four weeks post-injection with A β ₁₋₄₂ (Fig. 2F), indicating similar patterns of changes in both glutamate and aspartate. Parkin reversed the effects of A β ₁₋₄₂ on metabolic flux into aspartate isotopomers (Fig. 2E) and total pool size (Fig. 4F) compared to LacZ.

Lentiviral A β ₁₋₄₂ decreases GABA levels and induces oxidative stress

We further examined whether the changes in the excitatory amino acid neurotransmitter glutamate are associated with changes in inhibitory neurotransmitters, including β -amino butyric acid (GABA). Flux into GABA C2 was significantly decreased both two and four weeks post-injection with A β ₁₋₄₂ (Fig. 3A) compared to LacZ, but parkin reversed the decrease in flux back to control. Similarly, the total pool size of GABA was significantly decreased two and four weeks post-injection (Fig. 3B), while parkin reversed these effects.

To examine whether the changes in excitotoxic glutamate induce oxidative stress we determined whether A β ₁₋₄₂ expression results in lactate accumulation. No changes were observed in the flux of ¹³C label into lactate C3 two weeks post-injection, but flux was significantly increased ($P < 0.05$, $N = 5$) four weeks post-injection (Fig. 3C). The total level of lactate was also significantly increased at four, but not two weeks, post-injection (Fig. 3D), suggesting that lentiviral A β ₁₋₄₂ expression induces lactate accumulation, indicative of oxidative stress. However, parkin expression completely reversed flux into lactate C3 (Fig. 3C) and prevented lactate accumulation (Fig. 3D), indicating that parkin counteracts A β ₁₋₄₂-induced oxidative stress. Interestingly, glutamate levels were significantly increased prior to lactate accumulation, suggesting that glutamate toxicity may underlie A β ₁₋₄₂-induced oxidative damage even before other changes in amino acid levels.

To ascertain whether the changes in the level of by-products of the TCA cycle, including glutamate, glutamine, aspartate and GABA are associated with alteration in mitochondrial TCA cycle activity, we examined the levels of TCA intermediates succinate and citrate. No changes in metabolic flux into succinate C2/C3 or citrate C2 (Fig. 3C) were observed two weeks post-injection with lentiviral A β ₁₋₄₂, however, flux into citrate C2 was significantly decreased ($P < 0.05$, $N = 5$) four weeks post-injection. Similarly, no changes in total levels of TCA intermediates were observed at two weeks, but the levels of both succinate and citrate were significantly decreased four weeks (Fig. 3D), post-injection compared to LacZ control. Importantly, parkin expression reversed the decrease in metabolic flux into citrate C2 (Fig. 3C) and restored succinate and citrate levels (Fig. 3D) four weeks post-injection with A β ₁₋₄₂+parkin, suggesting that parkin protects against oxidative stress and mitochondrial impairment. These data suggest that parkin may protect against glutamate toxicity and mitochondrial impairment either via A β ₁₋₄₂ degradation (Burns et al., 2009, Khandelwal et al., 2011a) and/or by direct mitochondrial repair.

Parkin protects against brain atrophy in aged 3xTg-AD mice

To determine whether A β expression results in anatomical changes or atrophy in 3xTg-AD mice, we performed MRI and compared 1 year old non-Tg C57BL/6 (Fig. 4A) with age-matched 3xTg-AD mice (Fig. 4B). All animals were injected with lentiviral LacZ in the left hippocampus or parkin in the right hippocampus. No anatomical lesions or morphometric

differences were observed in these mice at one year of age. To quantify changes in brain volume we performed brain segmentation, which showed no noticeable differences in ventricular volume (Fig. 4E, N=11) and intracranial space (Fig. 4F, N=11) in 1 year old 3xTg-AD mice compared to age-matched control (Fig. 4E&F, N=17). However, 1.5 year old 3xTg-AD mice injected with lentiviral Lac-Z into the hippocampus showed observable cortical atrophy (Fig. 4C, arrowheads) and a significant increase (21%, N=11) in ventricular volume (Fig. 4C&E), but a non-significant increase (14%, $P<0.05$) in intracranial volume (Fig. 4C&F), compared to control (Fig. 4D, E and F). Importantly, expression of lentiviral parkin (N=11) for 3 months in the right hippocampus of 3xTg-AD mice was associated with absence of cortical atrophy and lack of changes in ventricular or intracranial volumes (Fig. 4C, E &F), suggesting that parkin protects against brain atrophy. To ascertain that the change in ventricular volume was not due to edema induced by parkin expression in these mice, we performed voxel localized MRS *in vivo* in the entire hemisphere to detect changes in osmo-regulatory molecules (i.e. taurine). No changes were observed in the levels of taurine (N=11) in un-injected hemispheres compared to parkin-injected hemispheres (Fig. 4G), or between young and old 3xTg-AD mice (data not shown), suggesting absence of brain swelling.

Parkin decreases cortical atrophy in lentiviral A β ₁₋₄₂ injected rat models

To determine whether parkin expression protects against amyloid-induced effects, we imaged rat brains injected with lentiviral A β ₁₋₄₂ in the left side of the M1 primary motor cortex and parkin+A β ₁₋₄₂ in the right side (Burns et al., 2009). Expression of A β ₁₋₄₂ led to obvious cortical atrophy around the injected brain region (Fig. 5A, arrowheads) 3 months post-injection. Quantification of changes in brain volume via segmentation showed that lentiviral expression of A β ₁₋₄₂ was associated with a significant increase (27%, N=6) in intracranial volume (Fig. 5A&B). Changes in ventricular volume were also apparent as the A β ₁₋₄₂ injected brain hemisphere showed a significant decrease (74%, $P<0.05$, N=6) in ventricular volume compared to LacZ or parkin+A β ₁₋₄₂. Co-injection of lentiviral A β ₁₋₄₂+lentiviral parkin (Fig. 5A) prevented cortical atrophy and reversed intracranial and ventricular volume back to control levels (Fig. 5B), suggesting that parkin counteracts the effects of A β ₁₋₄₂ on brain atrophy in gene transfer animal models. The cortical atrophy observed was localized to the vicinity of lentiviral A β ₁₋₄₂ injection and evident in the frontal but not caudal side of the brain.

To determine whether parkin protects against the toxic effects of A β ₁₋₄₂, we examined possible changes in cortical cell number using cresyl violet and GFAP staining and performed stereological counting. First we determined the effects of parkin on A β ₁₋₄₂ expressing brains three months post-injection, so we performed Western blot to measure the levels of TNF- β and iNOS as markers of inflammation. A significant increase (53%, by densitometry) in the levels of TNF- β was observed in A β ₁₋₄₂ expressing animals, compared to LacZ or parkin injected brains (Fig. 5C). Co-expression of parkin with A β ₁₋₄₂ was associated with decreased TNF- β levels back to control (LacZ). The levels of iNOS were also significantly increased (21% by densitometry, N=6) in A β ₁₋₄₂ injected animals compared to LacZ, parkin or parkin+A β ₁₋₄₂. We then performed immuno-histological staining to perform cell counting and ascertain whether brain atrophy is associated with cell loss in A β ₁₋₄₂ expressing cortex. Low view magnification showed decreased Cresyl violet staining (Fig. 5E, circle) in A β ₁₋₄₂ expressing cortex compared to parkin alone (Fig. 5D) or A β ₁₋₄₂+parkin (Fig. 6F). Stereological counting of cresyl violet positive cells revealed a significant (67%, $P<0.05$, N=11) decrease in cell count in A β ₁₋₄₂ injected cortical areas compared to both parkin and A β ₁₋₄₂+parkin. Higher view magnification show changes in cell morphology in A β ₁₋₄₂ injected cortex (Fig. 5H) compared to parkin (Fig. 5G) and A β ₁₋₄₂+parkin (Fig. 5I) injected animals. No differences were observed in the morphology

and number of astrocytes in parkin-injected (Fig. 3C) compared to LacZ injected (data not shown) controls (Rebeck et al., 2010). Lentiviral injection of A β ₁₋₄₂ resulted in pronounced changes of astrocyte morphology, suggesting astrogliosis (Fig. 5K), compared to parkin (Fig. 5J) and A β ₁₋₄₂+parkin (Fig. 5L) injected animals. Stereological counting of GFAP stained astrocytes revealed a significant increase (54%, N=6) in the number of astrocytes in A β ₁₋₄₂ compared to parkin injected brains. Co-injection of parkin and A β ₁₋₄₂ reversed A β ₁₋₄₂ effects on astrocyte morphology (Fig. 5L) and decreased the number of astrocytes back to control levels.

Discussion

These studies demonstrate that A β ₁₋₄₂ activates mTOR signaling, leading to increased phosphorylation of 4E-BP and translational initiation, which is controlled by the interaction between eIF-4E and 4E-BPs (Gingras et al., 1999, Gingras et al., 2001). Once 4E-BP1 is phosphorylated, it dissociates from eIF-4E, hence permitting cap-dependent translation to take place (Holz et al., 2005, Peterson and Sabatini, 2005). These mechanistic effects of A β ₁₋₄₂ suggest that mTOR signaling may be regulated by amino acid synthesis, including leucine, isoleucine and glutamine (Avruch et al., 2006). A β ₁₋₄₂ increased the synthesis of glutamate and aspartate, but not glutamine, leucine and isoleucine two weeks post-injection when no mTOR activity was detected (data not shown), but leucine and isoleucine levels increased while glutamate, glutamine and aspartate levels decreased four weeks post-injection with A β ₁₋₄₂. The current studies also demonstrate that lentiviral A β ₁₋₄₂ impairs mitochondrial TCA cycle and leads to oxidative stress, attenuating TCA activity and synthesis of its by-products, including glutamate, GABA, glutamine and aspartate. Two weeks after A β ₁₋₄₂ injection excitatory amino acids glutamate and aspartate were increased, while inhibitory GABA was decreased suggesting perturbation of neurotransmitter balance. However, 4 weeks post-injection all amino acid neurotransmitters were decreased, suggesting decreased production via TCA cycle metabolism. Additionally, A β ₁₋₄₂ induced lactate production, indicating oxidative stress along with mitochondrial impairment and neurotransmitter imbalance four weeks post-injection. Impaired activities of TCA cycle enzyme complexes pyruvate dehydrogenase, isocitrate dehydrogenase, and β -ketoglutarate dehydrogenase are found in post-mortem AD brains (Bubber et al., 2005), and in cells from AD patients (Huang et al., 2003). APP over-expression increases reactive oxygen species (ROS) levels, decreases mitochondrial membrane potential, and reduces ATP production in APPwt and APP^{swe} M17 cells (Wang et al., 2008). However, parkin reversed A β ₁₋₄₂ effects on TCA cycle in A β ₁₋₄₂-expressing animals, consistent with previous findings (Khandelwal et al., 2011a). The increase in glutamate levels prior to lactate accumulation suggests that glutamate toxicity may cause mitochondrial damage and lead to oxidative stress as a result of A β ₁₋₄₂ expression. Importantly, parkin reversed the effects of A β ₁₋₄₂ on amino acid levels, protected against glutamate toxicity and prevented mitochondrial TCA cycle impairment. Our data are consistent with previous findings suggesting that parkin expression reduces oxidative stress (Hyun et al., 2002), while blocking parkin expression increases oxidative damage (Palacino et al., 2004, Greene et al., 2005). The effects of parkin on markers of oxidative stress may be a result of parkin's role in mitochondrial function as parkin knockout transgenic mice (Palacino et al., 2004) and flies (Greene et al., 2003) have deficient mitochondria. Furthermore, several findings demonstrate that parkin is associated with mitophagy following loss of mitochondrial membrane potential (Matsuda and Tanaka, 2009, Chin et al., 2010). Therefore, parkin may protect against A β ₁₋₄₂ effects on alterations of brain metabolism via proteasome-autophagy degradation of amyloid and modulation of mitochondrial dynamics.

The present data suggest that parkin expression can counter A β effects, which may result in brain atrophy in animal models of AD. The 3xTg-AD mice display accumulation of

intraneuronal A β at 3 months, Tau accumulation at 6 months and extracellular plaques no earlier than 9 months of age (Oddo et al., 2003a, Billings et al., 2005, Oddo et al., 2006). Brain pathologies are also associated with cognitive defects in these mice (Oddo et al., 2003a, Oddo et al., 2003b). Despite the accumulation of several pathogenic proteins, 3xTg-AD mice do not have anatomical brain lesions or obvious morphological changes at least up to 1 year of age. Aging and protein accumulation result in overall brain atrophy, leading to increased ventricular volume and intracranial space. To determine the effects of amyloid proteins on brain atrophy, we injected lentiviral A β ₁₋₄₂, which contains a signal peptide directed to the endoplasmic reticulum to follow the secretory pathway of A β (Rebeck et al., 2010), and observed cortical atrophy 3 months post-injection. Amyloid expression was also associated with increased levels of inflammatory markers, neuronal loss and astrogliosis, suggesting cell death as we previously reported in gene transfer animal models (Burns et al., 2009, Rebeck et al., 2010, Khandelwal et al., 2011b). Parkin effects on A β levels may result in prevention of brain atrophy caused by amyloid accumulation in 3xTg-AD mice or A β ₁₋₄₂ expression in gene transfer animal models. Parkin protects against A β -induced cell death (Burns et al., 2009, Khandelwal et al., 2011b), and our current data show that parkin reverses the effects of A β on astrocytes and decreases the levels of inflammatory markers, including TNF- β and iNOS. Brain atrophy is largely recognized in AD in association with protein accumulation, which is progressively detected in the human brain, leading to transition from mild cognitive impairment (MCI) to advanced stages of disease pathology (Braak et al., 2006, Alafuzoff et al., 2008, Granic et al., 2010, Sabbagh et al., 2010, Murray et al., 2011). It is important to note the differential changes in ventricular volume in our animal models. The 3xTg-AD mice develop slow pathology with little cell loss and no measurable atrophy up 1 year of age compared to wild type controls. However, these mice develop observable cortical atrophy at 1.5 year of age, in association with significant ventricular enlargement while parkin abrogates atrophy, perhaps due to amyloid clearance (Khandelwal et al., 2011b). The lack of change in taurine levels, which is an osmo-regulator affected by brain swelling (Law, 2005, Ritz et al., 2006, Pascual et al., 2007), suggests that the difference between ventricular volumes is not due to edema caused by parkin expression, which also dampens gliosis. In contrast, the gene transfer animal models develop rapid degeneration due to expression of lentiviral A β ₁₋₄₂ and display Tau hyper-phosphorylation and gliosis within a focal region or the vicinity of lentiviral expression (Rebeck et al., 2010), suggesting that the atrophy observed in the frontal, not caudal part, of the cortex may lead to collapse of ventricles, while parkin clearance of A β ₁₋₄₂ prevents localized cortical atrophy and preserves ventricular integrity. In contrast, the A β transgene was globally expressed in 3xTg-AD animals, leading to generalized atrophy and enlargement of the ventricles. The choice of two animal models, including 3xTg-AD and lentiviral gene transfer was very useful to delineate the effects of A β and Tau accumulation in later stages (3xTg-AD) of AD-pathology compared to the early effects of A β ₁₋₄₂ (lentiviral gene transfer) on brain metabolism and morphometric changes.

In conclusion, the longitudinal studies suggest that AD genes alter brain metabolism and may induce slow atrophy in the brain of 3xTg-AD mice and lentiviral delivery indicates that A β ₁₋₄₂ causes astrogliosis and localized morphometric changes in the cortex of gene transfer animal models. Parkin expression reverses the effects of AD genes on inflammation and brain atrophy. Taken together these data suggest that parkin activity may be used as an alternative strategy to facilitate A β clearance via cellular quality control systems, including both the ubiquitin-proteasome and the autophagy lysosome pathways, thus limiting neurodegenerative death and subsequent brain atrophy in AD.

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Abbreviations

AD	Alzheimer's disease
APP	amyloid precursor protein
Aβ	β -amyloid
mTOR	mammalian target of Rapamycin
TNF-α	tumor necrosis factor- α
iNOS	inducible nitric oxide synthase
GFAP	glial fibrillary acidic proteins
eIF-4E	eukaryotic initiation factor 4E
4E-BP	eukaryotic initiation factor 4E binding protein
Glu	glutamate
Gln	glutamine
Cit	citrate
GABA	γ -amino butyric acid
Asp	aspartate
Leu	Leucine
Isoleuc	Isoleucine
Succ	succinate
Lac	Lactate
Lv	lentivirus
3xTg-AD	triple transgenic AD
ROS	reactive oxygen species

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Highlights

- 1- $A\beta_{1-42}$ increases glutamate and aspartate, but not glutamine levels
- 3- Glutamate levels increased prior to lactate accumulation
- 2- $A\beta_{1-42}$ attenuated mitochondrial TCA cycle and parkin reversed these effects
- 4- Parkin protected against $A\beta_{1-42}$ effects on amino acid and glutamate toxicity
- 5- Parkin reduced astrogliosis and reversed cell loss and cortical atrophy

Figure 1. Aβ₁₋₄₂ expression activates mTOR 4E-BP signaling

A). Histograms represent ELISA measurement of mTOR activity in immunoprecipitated cortical lysates. **B**). Western blot analysis of cortical brain lysates on 10% SDS NuPAGE gel showing total mTOR levels (1st blot) phosphorylated mTOR at Ser 2448 (2nd blot) and total p70S^{6k} (3rd blot) and phosphorylated p70S^{6k} levels at Thr 389 (4th blot) compared to actin control. **C**). Western blot analysis of cortical brain lysates on 4–12% SDS NuPAGE gel showing total 4E-BP1 levels (1st blot) phosphorylated 4E-BP1 at Thr 37/46 (2nd blot) and phosphorylated eIF-4E at Ser 209 (3rd blot) compared to actin control. Asterisk indicates significantly different, ANOVA, Mann Whitney, $P < 0.05$, $N = 8$.

Figure 2. High frequency $^{13}\text{C}/^1\text{H}$ NMR spectroscopy shows alterations of amino acid levels and parkin restores neurotransmitter balance

Histograms represent **A**). Flux of ^{13}C label from glucose into leucine C4 (Leu) and isoleucine C4 (Isoleu) and **B**). Pool size of total leucine and isoleucine in rat hemispheres injected with lentiviral parkin, parkin+Aβ₁₋₄₂, Aβ₁₋₄₂ or LacZ. **C**). Flux of ^{13}C label into glutamate C4 (Glu) and glutamine C4 (Gln) and **D**). Pool size of total glutamate and glutamine. **E**). Flux of ^{13}C label into aspartate C2 and C3 (Asp) and **F**). Pool size of total aspartate. * indicates significantly different, ANOVA, Mann Whitney, $P < 0.05$, $N = 5$.

Figure 3. High frequency $^{13}\text{C}/^1\text{H}$ NMR spectroscopy shows decreased GABA and TCA cycle activity and parkin counteracts oxidative stress
 Histograms represent **A**). Flux of ^{13}C label from glucose into GABA C2 and **B**). Pool size of total GABA in rat hemispheres injected with lentiviral parkin, parkin+Aβ₁₋₄₂, Aβ₁₋₄₂ or LacZ. **C**). Flux of ^{13}C label into lactate C3 (Lac), succinate C3 (Succ) and citrate C2 (Cit) and **D**). Pool size of total lactate, succinate and citrate. * indicates significantly different, ANOVA, Mann Whitney, $P < 0.05$, $N = 4$.

Figure 4. Parkin reduces brain atrophy in 3xTg-AD mice

A). MRI brain scans of 1 year old 3xTg-AD mice and, **B).** and 1 year old non-Tg (control) mice. **C).** MRI brain scans of 3xTg-AD mice injected with lentiviral parkin in the right hemisphere, compared to LacZ-injected left hemisphere. Arrowheads indicate cortical brain atrophy. **D).** MRI brain scans of 1.5 year old non-Tg control mice. Histograms represent quantification by segmentation of **E).** ventricular and **F).** intracranial volumes. **G).** Spectra obtained by MRS showing Taurine (Tau) levels. Bars are mean $\pm$ SD, N=11 each treatment, P<0.05, ANOVA Mann Whitney. Lv: Lentivirus. Asterisk indicates significantly different to all other conditions.

Figure 5. Parkin reverses cortical atrophy in $A\beta_{1-42}$ gene transfer animal models

A). MRI scans of rat brain injected with lentiviral $A\beta_{1-42}$ +lentiviral LacZ in the left hemisphere and lentiviral $A\beta_{1-42}$ +lentiviral parkin in the right hemisphere. Arrowheads indicate cortical atrophy. **B).** Histograms represent quantification of intracranial and ventricular volume. **C).** Western blot analysis of the levels of inflammatory markers TNF- β and iNOS in the cortex of gene transfer animal models (N=6). Cortical 20 μ m thick sections showing cresyl violet (Nissl) staining in lentiviral **D).** parkin, **E).** $A\beta_{1-42}$, circle indicates cell loss and **F).** parkin+ $A\beta_{1-42}$ injected brains. Higher view magnification show cell morphology in **G).** parkin, **H).** $A\beta_{1-42}$ and **I).** parkin+ $A\beta_{1-42}$ injected brains. Anti-GFAP immunoreactivity in brains injected with lentiviral **J).** parkin, **K).** $A\beta_{1-42}$ and **L).** $A\beta_{1-42}$ +parkin. Bars are mean $\pm$ SD, N=6 each treatment, P<0.05, ANOVA Mann Whitney. Lv: Lentivirus. Asterisk indicates significantly different to all other conditions.