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Increased AD-like pathology in the APP/ PS1 E9 mouse model lacking Nrf2 through modulation of autophagy

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Abstract

The presence of senile plaques is one of the major pathological hallmarks of the Alzheimer's disease (AD) brain. The plaques predominantly contain insoluble amyloid β -peptide; a cleavage product of the larger amyloid precursor protein (APP). Two enzymes named β and γ secretase generate the neurotoxic amyloid- β peptide from APP. Mature APP is also turnovered endogenously by autophagy, more specifically by the endosomal-lysosomal pathway. A defective lysosomal system is known to be pathogenic in AD. Modulation of NF-E2 related factor 2 (Nrf2) has been shown in several neurodegenerative disorders and Nrf2 has become a potential therapeutic target for various neurodegenerative disorders including AD, Parkinson's disease, and amyotrophic lateral sclerosis. In the current study, we explored the effect of genetic ablation of Nrf2 on APP/A β processing and/or aggregation as well as changes in autophagic dysfunction in APP/PS1 mice. There was a significant increase in inflammatory response in APP/PS1 mice lacking Nrf2. This was accompanied by increased intracellular levels of APP, A β (1-42), and A β (1-40), without a change total full-length APP. There was a shift of APP and A β into the insoluble fraction, as well as increased poly-ubiquitin conjugated proteins in mice lacking Nrf2. APP/PS1-mediated autophagic dysfunction is also enhanced in Nrf2 deficient mice. Finally, neurons in the APP/PS1/Nrf2^{-/-} mice had increased accumulation of multivesicular bodies, endosomes and lysosomes. These outcomes provide a better understanding of the role of Nrf2 in modulating autophagy in an AD mouse model and may help design better Nrf2 targeted therapeutics that could be efficacious in the treatment of AD.

Introduction

Alzheimer's disease (AD) is pathologically characterized by neuritic plaques consisting of extracellular amyloid- β (A β), and intracellular neurofibrillary tangles (NFT). There are two

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major β -amyloid precursor protein (APP) processing pathways. The amyloidogenic pathway generates the 39-43 amino acid A β peptides by the sequential action of β - and γ -secretase on the C-terminal of APP (Haass, 2004). In the non-amyloidogenic pathway, APP is processed by α -secretase instead of β -secretase to liberate sAPP α and generate α C-terminal fragment (Esch, et al., 1990, Sisodia, et al., 1990). APP is a short-lived protein that after maturation is rapidly internalized for either subsequent recycling or degradation by the endosomal-lysosomal pathway (Haass, et al., 1992).

NF-E2-related factor 2 (Nrf2) is implicated not only in mitigating oxidative stress and inflammation, both potentially key components in AD pathogenesis, but also in regulating directly and indirectly changes in autophagy *in vivo* and *in vitro* (Komatsu, et al., 2010, Lau, et al., 2010, Riley, et al., 2011, Riley, et al., 2010b). Nrf2 governs the expression of genes that are regulated by the antioxidant response element (ARE) of which several are phase II detoxifying enzymes or antioxidant proteins (Itoh, et al., 1997). Previous evidence suggests that Nrf2 activation can prevent or reduce cellular damage associated with injuries in different tissues and organs (Hybertson, et al., 2011, Taguchi, et al., 2011, Vargas and Johnson, 2009, Wakabayashi, et al., 2010). Genetically deleting Nrf2 exacerbates neuronal death in models of Parkinson's diseases (Innamorato, et al., 2010), Huntington's disease (Calkins, et al., 2005), Alexander's disease (Hagemann, et al., 2012) and aggravates experimental autoimmune encephalomyelitis, a model of multiple sclerosis (Johnson, et al., 2010). Additionally, Nrf2/ARE activation specifically in astrocytes confers protection to neighboring neurons in culture and *in vivo* (Chen, et al., 2009, Kraft, et al., 2004, Shih, et al., 2003, Vargas, et al., 2008). Previously, our laboratory has shown that astrocytic overexpression of Nrf2 extends lifespan, delays disease onset, and protects against motor neuron degeneration in mouse model of ALS (Vargas, et al., 2008). More recently, our laboratory showed that astrocytic Nrf2 activation dramatically delayed chaperone-mediated autophagy and macroautophagy dysfunction, onset of disease, and lifespan in a mouse model overexpressing the Parkinson's disease associated mutant human synuclein protein (hSYN^{A53T}) (Gan, et al., 2012).

Macroautophagy, hereafter referred as autophagy, mediates degradation of cytoplasmic constituents by sequestering them to double membrane vesicles called autophagosomes. Autophagy is important for neuronal survival (Mizushima, et al., 2008) and autophagy dysfunction has been identified and extensively studied in AD and several other neurodegenerative disorders [reviewed in (Nixon and Yang, 2012)]. The accumulation of both early phase and mature autophagic vacuoles (AVs) in dystrophic neurites in human AD brain suggests disrupted lysosome-autophagosome fusion and/or dysfunctional autolysosomal degradation that result in incomplete autophagic flux (Nixon and Cataldo, 2006, Nixon, et al., 2005). Indeed, autophagy is hypothesized to be a major mechanism mediating normal APP turnover, as well as a generator of intracellular A β when dysfunctional (Yu, et al., 2005). The lysosomal hydrolases responsible for APP processing in the lysosomal system have increased mRNA expression (Cataldo, et al., 1995) and are abnormally accumulated in A β plaques in early and late stages of AD pathogenesis (Bernstein, et al., 1992, Cataldo and Nixon, 1990, Cataldo, et al., 1990).

There is little work examining the role of Nrf2 in APP processing/turnover and autophagy in AD with no information existing in both animal models and human AD tissue. We hypothesized that lack of Nrf2 would aggravate pathologic changes observed in APP/PS1 mice by modulating autophagy, and tested this hypothesis by crossing APP/PS1 and Nrf2 deficient mice.

Materials and Methods

Mouse strains

APP(Swe)/PS1 E9 transgenic mice were purchased from The Jackson Laboratory [Strain Name: B6.Cg-Tg(APP^{swe},PSEN1^{dE9})85Dbo/J]. APP(Swe)/PS1 E9 mice coexpress human presenilin 1 (PS1) bearing the E9 deletion mutation and a chimeric mouse-human amyloid precursor protein (APP) 695 with mutations (K595N, M596L) linked to Swedish FAD pedigrees under control of the mouse prion protein promoter (Borchelt, et al., 1997, Borchelt, et al., 1996, Jankowsky, et al., 2001). The Nrf2^{-/-} mice were graciously provided by Drs. Kaiman Chan and Yuet Wai Kan (Howard Hughes Medical Institute, University of California, San Francisco, CA). The mice were created by replacement of the basic region-leucine zipper region with lacZ (Chan, et al., 1996). APP/PS1 heterozygous mice were maintained on C57BL/6J background. Nrf2^{-/-} mice were maintained on B6/SJL mice. Thus, these crossbred mice were on a mixed B6/SJL background. There were no significant pathological differences observed between non-transgenic and APP/PS1 mice on mixed strain versus the mice on C57/SJL pure strain mice at 7-month time point. Both male and female mice were used. Mice were group housed with food and water *ad libitum* and with a 12 h light/dark cycle. All the animal procedures were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. All the experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Genotyping

Genomic DNA was prepared from the tail biopsies and genotyped using PCR. Primers for Nrf2 are (5'-GGA ATG GAA AAT AGC TCC TGC C-3') and (5'-GCC TGA GAG CTG TAG GCC C-3') which gives a band at 262 bp in Nrf2 positive mice and a M13-40 primer designed against Lac X gene for Nrf2 deficient mice (5'-GGG TTT TCC CAG TCA CGA C-3') shows a band at 214 bp in Nrf2 deficient mice. Both the bands are observed in Nrf2 heterozygous mice (Fig S1a). The control primers for APP and PS1 are (5'-CCT CTT TCT GAC TAT GTG GAC TGA TG-3') and (5'-GTG GAT AAC CCC TCC CCC AGC CTA GA-3') which gives a band at 750 bp all the genotypes. The primers for APP transgene are (5'-GAC TGA CCA CTC GAC CAG GTT CTG-3') and (5'-CTT GTA AGT TGG ATT CTC ATA TCC-3') which gives a band at 350 bp in APP transgenic mice (fig S1b). The primers for PS1 transgene are (5'-GCC ATG AGG GCA CTA ATC AT-3') and (5'-ATT AGA GAA CGG CAG GAG CA-3') which gives a band at ~ 608 bp in PS1 transgenic mice (fig S1c).

Tissue Fractionation

The mice (n=3/group) were perfused with RNase-free PBS and hippocampus and cerebellum were collected. One-half of the tissue was flash frozen and used for fractionation followed by protein analysis. The other half was stored in RNAlater® (Life technologies) for future RNA extraction and analysis. The frozen tissue was thawed, weighed and processed by sonication in lysis buffer containing 1% Triton X-100, 50mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 1mM DTT, 1mM Na₃VO₄, 1mM NaF and protease inhibitor cocktail tablet from Roche as the ratio of 1:20 (1mg tissue = 20ul lysis buffer). The resulting total lysates were assayed for protein concentration by the Pierce BCA method (Bradford, 1976). To generate soluble and insoluble fractions, equal quantities of total lysates were centrifuged at 180,000g, 60min at 4°C. The supernatant was referred to as the triton-X soluble fraction. The pellets were washed with lysis buffer and then resuspended in equal volumes of buffer containing 30mM Tris-HCl, pH 8.5, 7M Urea, 2M thiourea and 4% CHAPS. The resuspended pellet was referred to as the triton-X insoluble or urea soluble fraction.

ELISA

Assay was performed as per manufacturers instruction using a human A β (1-42) ELISA kit (Invitrogen). Briefly, hippocampi were weighed and homogenized in 5M guanidine HCL and 50mM Tris HCL (pH 8.0) buffer and following mixing for 3-4h, samples were diluted at 1:50 ratio in ice cold reaction buffer containing 5% BSA, 0.03% Tween-20 and protease inhibitor cocktail (Roche) in 1X DPBS (pH 7.4) and centrifuged at 16,000g for 20 min at 4 deg C. Supernatant was carefully decanted and used with β -Amyloid ELISA kit from Invitrogen. 50 μ l of standards and samples were incubated overnight with 50 μ l of detection antibody per well at 4 deg C. Wells were washed and incubated with 100 μ l of HRP Anti-Rabbit antibody for 30 min at RT. following that the wells were washed and incubated with 100 μ l of stabilized Chromogen for 30 min at RT. Color development was visualized and 100 μ l of stop solution was added. The plate was read at 450nm on a UV-Visible plate reader.

Western blotting

Proteins were resolved on SDS–polyacrylamide gel. For A β western blot, triton-x soluble and urea soluble fractions were resolved on 10% Tris-Tricine polyacrylamide gel. The resolved proteins were transferred to Hybond-P membrane (Amersham). Membranes were blocked for 1 h in TBS, 0.1% Tween 20 and 5% non-fat dry milk, followed by an overnight incubation with primary antibody diluted in the same buffer. Membranes from A β western blot were boiled in 1X PBS (Lonza) prior to blocking. For phospho-specific proteins, membranes were blocked in TBS, 0.1% Tween 20 and 5% Bovine serum albumin (BSA) and overnight incubation in primary antibody diluted in same buffer. After washing with 0.1% Tween in Tris-buffered saline, the membrane was incubated with peroxidase-conjugated secondary antibody (antibody to Rabbit and Mouse IgG from KPL and Goat IgG from Santacruz biotechnologies) for 1 h, and then washed and developed using the ECL chemiluminescent detection system (Supersignal® west pico from Thermo Scientific) in Syngene G:Box using ichemiXT GENesys program. Densitometric analyses were

performed using the Gene tools from Syngene program and normalized against the signal obtained by reprobing the membranes with anti-Tubulin 1:10,000 (Clone E7; Developmental Studies Hybridoma Bank-University of Iowa). The dilutions for primary antibodies: 22C11 1:500 (Millipore), A β 1-16 (6E10; Covance) 1:1000, C-terminal fragment clone C1/6.1 1:1000 (Covance), CathepsinD (CatD) 1:2000 (Santa Cruz Biotechnology), NADPH:quinone oxidoreductase (Nqo1) 1:1000 (Epitomics), tubulin 1:5000 (Clone E7, DSHB, University of Iowa), Lamp2a 1:1000 (Abcam), Lamp1 1:1000 (Abcam), microtubule-associated protein light chain 3 1:1000 (LC3; Cell Signal). mTOR 1:1000 (Cell Signal), Phospho-mTOR 1:1000 (Cell Signal), p70S6K 1:1000 (cell Signal), phospho-p70S6K 1:1000 (cell Signal), ubiquitin (VU1) 1:1000 (Life Sensors), sequestosome 1 (SQSTM1, P62) 1:2000 (Abnova), glutamate-cysteine ligase catalytic subunit (GCLc) and modifier subunit (GCLm) 1:10000 (provided by Dr. Terry Kavanagh, University of Washington, Seattle, WA), GFAP 1:10,000 (Sigma), IBA1 1:500 (Dako).

Immunofluorescence and histochemistry

Mice (n=3/group) were perfused with PBS followed by 4% paraformaldehyde (PFA). Tissues were postfixed overnight in 4% PFA and run through dehydration followed by paraffin embedding program. Coronal sections (5 μ m) were prepared using a Microtome (Leica). Sections were deparaffinized and rehydrated followed by antigen retrieval using microwave irradiation in citrate buffer. The sections were permeabilized and blocked with 10% goat serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS for 1 h at room temperature (RT), then incubated with primary antibodies diluted in 10% goat serum, 1% BSA in PBS at 4°C overnight. The appropriate IgG was used for controls. The following day, sections were incubated with appropriate Alexa-Fluor secondary antibodies diluted in the same solution for 1 h at RT after intensive wash. The slides were coverslipped with Dapi-Fluoromount-G™ (SouthernBiotech) and post-drying were visualized under fluorescent microscope. For light microscopy, sections after antigen retrieval were processed using VECTASTAIN® Elite® ABC System as per manufactures protocol. The staining was visualized by DAB kit from Vector lab. Sections were counterstained with Hematoxylin QS (Vector labs) for visualizing nucleus. Primary antibodies were used at the following dilutions: Beta Amyloid, 1-16 (6E10), Beta Amyloid, 17-24 (4G8) 1:1000 (Covance), Anti-Beta-Amyloid 1-42 (AB5078P), Anti-Beta-Amyloid 1-40 (AB5074P) 1:100 (Millipore), Anti-Alzheimer precursor protein A4 (22C11) 1:100 (Millipore), Rabbit β -III-tubulin 1:1000 (Abcam), Mouse β -III-tubulin 1:1000 (Promega), GFAP 1:1000 (Dako), ionized calcium binding adaptor molecular (IBA1) 1:500 (Wako), p62 1:500 (Abnova), and ubiquitin (VU-1) 1:250 (Life Science), Lamp1 1:1000 (Abcam). Secondary antibodies used were Alex Fluor 488 and 647 (Invitrogen) at the dilution of 1:1000.

Real-time PCR

RNA was isolated using TRIZOL reagent (Life technologies) as per manufacturers instructions. Quality of RNA was assessed with the 2100 Bioanalyzer (Agilent Technologies) and 2 μ g RNA was reverse transcribed using OligoDT primers and Reverse transcription system kit (Promega) according to the manufacturer's protocol. Real time PCRs were performed in a 20 μ l reaction with 1X LightCycler480 SYBR Green I Master (Roche) containing 2 μ l of cDNA and 20pmol of each specific primer in a LightCycler480 Real-time

PCR System (Roche). The cycling parameters were as follows: 95°C, 10 s; 55°C, 10 s; 72°C, 15 s. Minus reverse transcriptase controls were included in each assay. Specific primers were:

Nrf2 Forward (5'-GTCTTCACTGCCCCTCATC-3'),
Nrf2 Reverse (5'-TCGGAATGGAAAATAGCTCC-3'),
Keap1 Forward (5'-AAGGACCTTGTGGAAGACCA-3'),
Keap1 Reverse (5'-CCCTGTCCACTGGAATTGAT-3')
Nqo1 Forward (5'-ACCTTGCTTTCTATCACCAGT-3'),
Nqo1 Reverse (5'-TGAATCGGCCAGAGAATGAC-3'),
HO-1 Forward (5'-GAAACCCAGATCAGCACTAG-3'),
HO-1 Reverse (5'-ATTGGACAGAGTTCACAGCC-3'),
GCLM Forward (5'-GCCACCAGATTTGACTGCCTTTG-3'),
GCLM Reverse (5'-TGCTCTTCACGATGACCGAGTACC-3'),
GCLC Forward (5'-ACATCTACCACGCAGTCAAGGACC-3'),
GCLC Reverse (5'-CTCAAGAACATCGCCTCCATTCAG-3'),
Gpx4 Forward (5'-AGGTCTGCCTGAAAACCAG-3'),
Gpx4 Reverse (5'-CATTTTATTCCCACAAGGCAGC-3'),
Prdx1 Forward (5'-GTGAGACCTGTGGCTCGAC-3'),
Prdx1 Reverse (5'-TGTCCATCTGGCATAACAGC-3'),
Aox1 Forward (5'-CTTTTGACCAAAGCATCAGTCTC-3'),
Aox1 Reverse (5'-CCCTTTCTCCCAGTCTATATTCGA-3'),
GSTA4 Forward (5'-ACCCTTGGTTGAAATCGATGG-3'),
GSTA4 Reverse (5'-CTTCAGGTCCTTCCCATAACAAG-3'),
GFAP Forward (5'-CAGACTTTCTCCAACCTCCAG-3'),
GFAP Reverse (5'-CATCTCCACAGTCTTACCACG-3'),
IBA1 Forward (5'-CGAATGCTGGAGAACTTGG-3'),
IBA1 Reverse (5'-AGAGTAGCTGAACGTCTCCT-3'),

p62 Forward (5'-TGTGGAACATGGAGGGAAGAG-3'),

p62 Reverse (5'-TGTGCCTGTGCTGGAACCTTTC-3'),

Actin Forward (5'-TCCTAGCACCATGAAGATCAAG-3'),

Actin Reverse (5'-GAAGGTGGACAGTGAGGC-3')

Electron microscopy

Transmission electron microscopy was performed as described previously (Gan, et al., 2012). Briefly, mice were perfused with PBS followed by 4% PFA. The brain was dissected and post-fixed in 2% glutaraldehyde and 2% PFA in PBS for overnight. The primary fixed samples were rinsed 5 times for 5 min in PBS, and post-fixed in 1% osmium tetroxide, 1% potassiumferrocyanide in for an hour at RT in PBS followed by rinsing in PBS. After dehydration, samples were infiltrated with the mixtures of PolyBed 812 (Polysciences Inc. Warrington, PA) and propylene oxide with increasing concentrations. The samples were sectioned on a Leica EM UC6 ultramicrotome at 90 nm, and collected on Cu, pioloform coated 2×1 oval slot grids (EMS Hatfield, PA) followed with poststaining in uranyl acetate and lead citrate. The sections from different genotypes of mice were viewed at 80 kV on a Philips CM120 transmission electron microscope, equipped with MegaView III camera (Olympus Soft Imaging System Lakewood, CO).

Statistical analysis

All data are represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni multiple paired comparisons post hoc test (GraphPad prism 4) ($p < 0.05$).

Results

Modest changes in the Nrf2 and Nrf2-dependent gene expression and protein levels in APP/PS1 mice

APP/PS1/Nrf2+/- (heterozygous) mice were crossed with Nrf2+/- mice to generate 6 possible genotypes. APP/PS1/Nrf2+/+ versus APP/PS1/Nrf2-/- mice were used for the experiments conducted. The mice were aged to 7-8 months and tissues were harvested. As seen previously in our laboratory (Kraft, et al., 2006), the Nrf2 deficient mice have minimal changes in the expression level of Nrf2-dependent genes (Fig 1A). Axl1 (aldehyde oxidase 1) was the only gene reduced in the Nrf2-/- mice. In regard to APP/PS1 mice, there were no significant changes in any of the genes evaluated on a Nrf2+/+ background. The APP/PS1/Nrf2-/- mice did have significant differences in heme oxygenase-1 (HO1; increase), glutathione peroxidase-4 (GPx4; increase), glutamate cysteine ligase modifier subunit (GCLM; increase) and Axl1 (decrease) relative to Nrf2-/- mice. To verify some of these modest changes in mRNA levels correlated with protein changes, Keap1, Nqo1, GCLM and glutamate cysteine ligase catalytic subunit (GCLC) were evaluated by western blotting. Again, there were small but significant changes in some of the proteins (GCLM and GCLC)(Fig. 1B, 1C)

Significant increase in inflammatory response in APP/PS1 mice lacking Nrf2

Based on previous studies in models of neurodegeneration, Nrf2^{-/-} mice have shown an increased neuroinflammatory response observed in disease states relative to Nrf2^{+/+} mice. This observation was consistent in the APP/PS1 mice. There was an increase in glial fibrillary acidic protein (GFAP) immunofluorescence in CA1 neuronal field in APP/PS1/Nrf2^{-/-} mice compared to littermate controls (Fig. 2A). Accordingly, a significant increase in GFAP mRNA expression and protein level was observed in APP/PS1/Nrf2^{-/-} mice (Fig. 2B, E and F). A similar pattern was seen for microglial activation using ionized calcium-binding adapter molecule 1 (IBA1) immunofluorescence (Fig 2C) and IBA1 mRNA expression and protein level in hippocampus of APP/PS1/Nrf2^{-/-} mice (Fig. 2D, E and F).

Lack of Nrf2 increases APP/A β levels in APP/PS1 mice

Plaques were visualized by anti-A β (1-16) 6E10 and anti-A β (17-24) 4G8 immunostaining (Fig. 1 A). There were no obvious changes in plaque load in APP/PS1/Nrf2^{-/-} mice compared to APP/PS1 mice (Fig 3A). A higher magnification image of CA1 neuronal field revealed an increased APP/A β levels in both 6E10 and 4G8 immunostained CA1 region from APP/PS1/Nrf2^{-/-} mice (Fig. 3Ac, Ad, Ag and Ah). Since 6E10 recognize full-length mouse and human APP along with A β , we fractionated the hippocampus into triton-X-soluble and urea-soluble fractions that were run out on a tris-tricine gel (Fig 3B). There was a significant increase in low molecular weight triton-X-soluble and urea-soluble A β oligomers in APP/PS1/Nrf2^{-/-} mice compared to APP/PS1 mice. There was also an increase in total A β levels in triton-X-soluble and urea-soluble fraction in the APP/PS1/Nrf2^{-/-} mice. A prominent A β *56 band was observed in all the 4 genotypes but its levels were higher in mouse bearing mutant APP/PS1. Interestingly, the A β *56 levels were higher in Nrf2^{-/-} and APP/PS1/Nrf2^{-/-} urea-soluble fraction compared to their respective littermates (Fig. 3B). The dodecamer form of A β is recognized by 6E10 antibody and previous studies demonstrate an increase in its levels in transgenic mouse model of AD (Larson and Lesne, 2012). Although it is not clear how the A β *56 oligomer is involved in neurodegeneration in AD but it has been predicted to be pathologic in human AD cases (Lesne, et al., 2013).

Absence of Nrf2 in APP/PS1 mice increases human A β (1-42) levels and A β (1-42) and A β (1-40)-specific immunostaining

The hippocampus tissue sections were stained with antibodies that specifically recognize A β (1-42) and (1-40) but does not recognize full-length APP. An increase in A β (1-42)-specific (Fig. 4a and b) and in A β (1-40)-specific immunostaining (Fig. 4c and d) in hippocampal region of APP/PS1/Nrf2^{-/-} mice compared to APP/PS1 mice. Furthermore, we quantified the total human A β (1-42) level in transgenic mice by ELISA. There was a significant increase in total human A β (1-42) levels in APP/PS1/Nrf2^{-/-} mice hippocampus compared to APP/PS1/Nrf2^{+/+}.

Lack of Nrf2 in APP/PS1 mice does not change total full-length APP levels but drives its accumulation into the urea-soluble fraction

In order to distinguish between A β and full-length APP levels, we used an antibody specific to APP that does not recognize A β (1-42/40) (22C11). There were no significant changes in total full-length APP levels in APP/PS1/Nrf2^{-/-} hippocampus compared to APP/PS1. In addition, no significant change in C-terminal fragments was observed in APP/PS1/Nrf2^{-/-} hippocampus (Fig. 5A,B,C). Even though there was no significant increase in total full-length APP, there was a clear increase in 22C11 immunoreactivity in hippocampus of APP/PS1/Nrf2^{-/-} mice (Fig. 5D). As expected based on previous publications (Lewis, et al., 2001), the 22C11 antibody weakly stains plaques (Fig. 5Da,b). A higher magnification image from the CA1 neuronal field showed an increase in 22C11 staining that appeared to be intraneuronal (Fig. 5Dc,d).

Based on the observation from the 22C11 immunostaining, we examined the triton-X-soluble and urea-soluble hippocampus fractions from APP/PS1/Nrf2^{-/-} and APP/PS1 mice on a tris-tricine gel. There was actually a decrease in the full-length APP levels in triton-X-soluble fraction and increased APP in urea-soluble fraction in APP/PS1/Nrf2^{-/-} mice (Fig. 6A and B). These data strongly suggest that there is a shift in solubility/clearance that lead to increased insoluble APP to accumulate in neurons and/or plaques. A longer exposure showed an increase in N-terminus APP fragments in APP/PS1/Nrf2^{-/-} in both triton-X-soluble and insoluble fractions (Fig. 6A and B). Interestingly, there was also a significant increase in insoluble (urea-soluble) endogenous mouse APP in the Nrf2^{-/-} mice without the overexpression of human APP and PS1 (Fig. 6B).

Lack of Nrf2 in APP/PS1 mouse modulates autophagy

The Sqstm1/p62 is a selective substrate of autophagy that targets ubiquitin conjugated proteins for degradation. During autophagy, p62 binds LC3-II (microtubule-associated light chain C II) while trafficking ubiquitinated (Ub) proteins for autophagosome-dependent degradation. When autophagy is impaired, p62 oligomers and Ub-containing proteins form aggregates/inclusions (Riley, et al., 2010b). There was a significant increase in p62 protein levels in APP/PS1 mice compared to WT mice without any change in mRNA levels (Fig. 7A, B and C). The opposite was observed in the APP/PS1/Nrf2^{-/-} mouse compared to APP/PS1 mouse where p62 was actually decreased without changing mRNA (Fig. 7A, B). Further analysis of triton-X- and urea-soluble fractions from hippocampus revealed a shift in p62 levels from the triton-X-soluble to insoluble pellet in APP/PS1 mice that was increased in the APP/PS1/Nrf2^{-/-} mice (Fig. 7A, C). This accumulation of insoluble p62 inclusion was further visualized by p62 immunofluorescence in APP/PS1 and APP/PS1/Nrf2^{-/-} mice (Fig. 7B) suggesting that autophagic clearance of p62 is impaired.

Autophagic flux is most commonly analyzed by determining the ratio of microtubule-associated 1A/1B-Light chain 3 I (LC3I) and LC3II. During autophagy the LC3I is conjugated to phosphatidylethanolamine to form LC3II. The LC3II is then recruited to autophagosomal membranes during nucleation/maturation stage of autophagosome formation. Hence, the ratio of LC3II to LC3I serves as an indicator of autophagic activity. There was significant increase in LC3BII to LC3BI ratio in APP/PS1 and APP/PS1/Nrf2^{-/-}

mice hippocampus compared to their respective littermate controls (Fig. 7D, E). This APP/PS1-associated increase in the LC3II/LC3I ratio was not altered in mice lacking Nrf2 that have the APP/PS1 transgene (Fig. 7D, E).

Absence of Nrf2 in APP/PS1 mouse leads to the increased accumulation of poly-ubiquitin conjugated proteins

The above-mentioned shift in solubility of p62 in APP/PS1 mouse lacking Nrf2 was accompanied by accumulation of poly-ubiquitin conjugated protein. There was a significant increase in poly-ubiquitin conjugated protein in triton-X-soluble and urea-soluble fraction from APP/PS1/Nrf2^{-/-} hippocampus compared to its littermate control as observed by VU1 immunoblot (Fig. 8A, B). The poly-ubiquitin conjugated protein were also visualized as inclusion in CA1 neurons and an overall increase in VU1 immunostaining in CA1 field was observed in APP/PS1/Nrf2^{-/-} mice compared to APP/PS1 mouse (Fig. 8C).

Absence of Nrf2 in APP/P1 mouse leads to autophagy inhibition

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates several cellular functions including, but not limited, to autophagy. One of the downstream effectors of mTOR pathway is p70S6 kinase (p70S6k), which is also shown to phosphorylate mTOR at Ser-2448 (Chiang and Abraham, 2005). Activation of the mTOR pathway inhibits autophagy. In APP/PS1/Nrf2^{-/-} mice hippocampus, a significant increase in p-mTOR/total mTOR ratio was observed (Fig. 9A, B). This change in mTOR activation correlated with an increased p-p70S6k/total p70S6k ratio in the APP/PS1/Nrf2^{-/-} mice (Fig. 9C, D). The observations are indicative of autophagy inhibition.

Furthermore, an increased 6E10 immunostaining was observed in APP/PS1/Nrf2^{-/-} mice CA1 neurons compared to APP/PS1, which co-localized with lysosomal protein Lamp1 in the cells (Fig. 10A). We measured the two major lysosomal proteins, Lamp1 and Lamp2a by western blot (Fig. 10B). Although there was a significant increase in total Lamp1 protein levels in APP/PS1 mice hippocampus, it was not changed significantly on a Nrf2^{-/-} background (Fig. 10B). However, a significant decrease to almost same extent in Lamp2a protein was observed in both APP/PS1 and APP/PS1/Nrf2^{-/-} mice compared to their respective littermate controls (Fig. 10B). The Lysosomes contain acid hydrolases that break down the cellular components either in autolysosomes or endolysosomes. Cathepsin D is one such aspartic protease that critically depends on protonation of active form to function, hence requiring lower pH levels inside the lysosomes for its activity. There was a small, but not significant, increase in mature cathepsin D in APP/PS1/Nrf2^{-/-} hippocampus compared to APP/PS1 mice and other littermate controls (Fig. 10C). However, a significant increase in pre-cathepsin D was observed in hippocampus from APP/PS1/Nrf2^{-/-} compared to APP/PS1 mice (Fig. 10C) suggesting dysfunctional maturation of pre-cathepsin D to its active form.

Several lines of evidence suggest that autophagy inhibition leads to accumulation of vacuoles, vesicles involved in degradation of cytoplasmic components, specifically in neurons from AD mouse models (Nixon, et al., 2005, Nixon and Yang, 2011, Nixon and Yang, 2012). Further evidence of involvement of Nrf2 in autophagy in AD mouse model

comes from TEM images (Fig. 11). There was multivesicular body accumulation in APP/PS1 mouse on Nrf2^{+/+} background (denoted by arrows in Fig. 11A,C) as observed previously by others. Interestingly, lack of Nrf2 further increased the accumulation of multivesicular bodies, endosomes and lysosomes that are involved in degradation of APP and other cytoplasmic components (Fig. 11B,D). Finally, CA1 neurons in APP/PS1/Nrf2^{-/-} mice had aberrant cytoplasmic constituents and were surrounded by dystrophic or dead cells. These anomalous cells were found with much greater frequency in APP/PS1/Nrf2^{-/-} hippocampus compared to APP/PS1/Nrf2^{+/+} hippocampus (Fig. 11E,F).

Discussion

Studies from human AD brain suggest an increase in Nqo1 activity in vulnerable neuronal populations as well as astrocytes (Raina, et al., 1999, Wang, et al., 2000). Additionally, a significant increase in HO-1 expression was reported in post-mortem AD temporal cortex and hippocampus compared to aged-matched control (Schipper, et al., 2006). Studies also show that Nrf2 is predominantly localized to the cytoplasm in AD hippocampal neurons (Ramsey, et al., 2007). In a triple transgenic AD mouse, the GSH/GSSG ratio was reported to be reduced indicating a shift in redox balance towards to more oxidative state in these mice compared to the non-transgenic control mice (Resende, et al., 2008). In aged APP/PS1 mice, Nrf2, Nqo1, GCLC, GCLM mRNA and Nrf2 protein levels were reduced compared to aged matched control (Kanninen, et al., 2008). Contrary to these observations, our data suggest that there is no change in Keap1, Nrf2 or any of the Nrf2-dependent genes examined in APP/PS1 compared to age-matched controls. There were, however, modest decreases and increases associated with APP/PS1 on a Nrf2^{-/-} background. Some small but significant changes were also observed at the protein level (Fig. 1). Overall, we would conclude that the Nrf2 pathway is not dramatically affected in APP/PS1 mice. In contrast, there was an enhanced astrogliosis and microgliosis on the Nrf2^{-/-} background suggesting that there may be an increased pathology in the Nrf2^{-/-} (Fig. 2). Indeed, APP processing and clearance were altered on the Nrf2^{-/-} background.

APP, on the cell surface, is internalized rapidly within early endosomes, where cleavage from β -secretase (BACE) releases a soluble APP fragment (sAPP β) and generates a membrane-associated 99-residue C-terminal fragment (β -CTF) that contains the whole A β peptide. The β -CTF is further processed by the γ -secretase complex generating A β . The components of the β -secretase complex have been found in endosomes (Capell, et al., 2000, Vassar, et al., 1999, Walter, et al., 2001) and trans-golgi network (von Arnim, et al., 2006), whereas components of γ -secretase complex have been detected in many cellular locations, including at the plasma membrane and in early endosomes (Runz, et al., 2002), late endosomes, autophagic vacuoles (Yu, et al., 2005, Yu, et al., 2004) and lysosomes (Cupers, et al., 2001, Pasternak, et al., 2003). Several studies have reported that processing of APP in the endosomal-lysosomal pathway is source of intracellular A β (Cataldo, et al., 2004, Takahashi, et al., 2004). Our data suggest a significant increase in what appeared to be more intracellular 6E10 and 4G8 immunostaining in CA1 neurons in APP/PS1/Nrf2^{-/-} mouse compared to APP/PS1. Although, there was no visible difference in plaque load in APP/PS1/Nrf2^{-/-} mouse compared to APP/PS1 mouse (Fig. 3), but we saw a significant increase in total human A β (1-42) levels in hippocampus of APP/PS1/Nrf2^{-/-} mouse

compared to APP/PS1 mouse (Fig. 4B). Since 6E10 and 4G8 recognize both A β and full-length APP, it was critical to differentiate between the two to clarify if the observed increase in immunostaining was coming from A β or APP. We used antibodies specific for A β (1-42), A β (1-40) and full-length APP (22C11). Undeniably, there was increase in A β -specific immunostaining in hippocampus of APP/PS1/Nrf2^{-/-} compared to APP/PS1 mouse (Fig. 4A). The full-length APP-specific (22C11) immunostaining was also increased in hippocampus, and plaques in hippocampus and CA1 neuronal field (Fig. 5D). There was no significant trend towards increase in CTFs (β -, and γ -CTF) or levels of total full-length APP in hippocampus of APP/PS1/Nrf2^{-/-} compared to APP/PS1. However, when the triton-X-soluble and urea-soluble fraction from hippocampus were probed for 22C11 on an immunoblot, a decrease in the soluble APP and an increase in the insoluble APP was observed in APP/PS1/Nrf2^{-/-} suggesting a shift in APP solubility in APP/PS1/Nrf2^{-/-} mouse. Thus, it appears that APP has accumulated in the intracellular milieu and/or extracellular plaques. These data also suggest that, even though there is no change in synthesis and maturation of APP, the processing and/or clearance is altered in APP/PS1/Nrf2^{-/-} mouse. We did not observe any change in either BACE1 or PS1-N-terminal fragment protein levels in hippocampus from APP/PS1/Nrf2^{-/-} and APP/PS1 mouse (data not shown). This does not rule out the fact that the activity of either BACE or PS1 may have been altered. More cohorts are being generated to further investigate the properties of APP processing enzymes.

It has been suggested that Nrf2 is involved in regulation of autophagy via increasing p62 (Fujita, et al., 2011, Jain, et al., Riley, et al., 2010a). Most recently, our laboratory has shown that Nrf2 in astrocytes delayed chaperone-mediated autophagy and macroautophagy dysfunction in the hSYN^{A53T} mice suggesting that Nrf2 in astrocytes provides neuroprotection against neuronally overexpressed hSYN^{A53T}-mediated toxicity by promoting the degradation of hSYN^{A53T} through the autophagy-lysosome pathway *in vivo* (Gan, et al., 2012). Recent studies from human AD brain revealed that p62 interacts with Keap1 and accumulates as cytoplasmic inclusions in neurons and glial cells (Tanji, et al.). The data from our AD mouse model showed a significant increase of p62 protein levels in APP/PS1 compared to wildtype controls (Fig. 7A,C). The lack of Nrf2 did not change p62 levels, however, in the presence of APP/PS1 on a Nrf2^{-/-} background, the level of p62 actually was decreased as demonstrated by western blots from hippocampal tissue (Fig. 7A,C). The p62 immunofluorescence showed a more diffused staining pattern in APP/PS1 CA1 neurons, whereas, a more punctate staining that looked like inclusion body in APP/PS1/Nrf2^{-/-} CA1 neurons (Fig. 7B) implying that the lack of Nrf2 in APP/PS1 mice drives p62 into more insoluble aggregates. Indeed, there was an increase in urea-soluble and decrease in triton-X-soluble p62 levels in APP/PS1/Nrf2^{-/-} hippocampus compared to APP/PS1 mice rendering it dysfunctional. Apart from binding Keap1 and LC3II, p62 also binds to poly-ubiquitinated proteins and target them for subsequent degradation. We observed an increase in ubiquitinated protein in both the soluble and insoluble fraction from hippocampus of APP/PS1/Nrf2^{-/-} compared to APP/PS1 mouse that correlates with reduced p62 levels in APP/PS1/Nrf2^{-/-} hippocampus (Fig. 8). Immunofluorescence revealed VU1 positive inclusion in CA1 neurons from APP/PS1/Nrf2^{-/-} suggesting accumulation of ubiquitinated protein. In addition, there was an increase in autophagosome

formation as accessed by the LC3II to LC3I ratio in both APP/PS1 and APP/PS1/Nrf2^{-/-} hippocampus with a trend (not significant) towards increase in APP/PS1/Nrf2^{-/-} hippocampus (Fig. 7D,E).

Autophagy induction is regulated by the mTOR pathway, which suppresses autophagy in response to insulin signaling and/or elevated nutrient levels (Meijer and Codogno, 2006). Studies from *in vitro* and *in vivo* mouse models and human AD cases suggest that mTOR signaling pathway is hyperactive in AD (Oddo, 2012). N2a cells treated with A β had higher expression of p70S6k, a downstream target of mTOR, and increased levels of phosphorylated p70S6k correlates with increased neurofibrillary tangle pathology in AD cases (An, et al., 2003, Lafay-Chebassier, et al., 2005). Additionally, Chinese hamster ovary cells stably transfected with a familial APP mutation (7P2A cells) had higher mTOR activity compared to normal cells (Caccamo, et al., 2010). Studies from animals show an increase in mTOR activity and downstream p70S6k levels of protein in cortex and hippocampus of mouse models of AD (Caccamo, et al., 2010, Caccamo, et al., 2011). Furthermore, injection of oligomeric A β in hippocampus of wild type mice increased mTOR activity (Caccamo, et al., 2011). Consistent with these studies, there was an increase in the ratio of p-mTOR/total mTOR, as well as, an increase in the ratio of p-p70S6k/total p70S6k in hippocampus of APP/PS1/Nrf2^{-/-} mice compared to littermate controls indicative of autophagy inhibition (Fig. 9). The mTOR signaling pathway activation may be in response to increased A β /APP accumulation or increased inflammatory response in APP/PS1 mice lacking Nrf2. The activation of mTOR also correlates with the increased poly-ubiquitin conjugated proteins in APP/PS1/Nrf2^{-/-} mice hippocampus.

Finally, we investigated the lysosomal system and found that there was no significant difference in lysosomal protein Lamp1 and Lamp2a between APP/PS1 and APP/PS1/Nrf2^{-/-} hippocampus. However, immunofluorescence from CA1 neurons revealed that there was increased 6E10 immunoreactivity in APP/PS1/Nrf2^{-/-} mice that co-localized with Lamp1 compared to APP/PS1 mice suggesting accumulation of A β /APP in lysosomal vesicles (Fig. 10). This may have resulted because of autophagy inhibition in APP/PS1/Nrf2^{-/-} mice hippocampus. Furthermore, there was accumulation of premature cathepsin D, an important lysosomal hydrolase, in APP/PS1/Nrf2^{-/-} mice (Fig. 10). More evidence of autophagy inhibition in Nrf2 lacking APP/PS1 mouse comes from TEM images (Fig. 11). An increase in multivesicular bodies, endosomes and lysosomes was observed in CA1 neuron of APP/PS1/Nrf2^{-/-} mouse compared to APP/PS1 mouse CA1 neuron. Autophagy failure in AD has been ascribed to the inhibition of the lysosomal degradation following accumulation in dystrophic neuritis (Nixon and Yang, 2011). Additionally, autophagy failure has been attributed to defective lysosomal acidification and reduced maturation/activity of lysosomal hydrolases (Wolfe, et al., 2013). Independent of γ -secretase function, PS1 is shown to be involved in lysosomal acidification that is required for maturation and activation of lysosomal hydrolases such as cathepsin B and D (Lee, et al., 2010, Wolfe, et al., 2013). In APP/PS1/Nrf2^{-/-} mice, perhaps lysosomal acidification is not occurring that prevented the maturation of pre-cathepsin D leading to incomplete processing of APP/A β and APP/A β accumulation in the lysosomes. A further investigation into the detailed mechanism leading to these observations and analysis of behavioral deficits, if any, is required, which is focus

of our ongoing experiments. However, the data obtained from AD transgenic mice merits the role of Nrf2 in disease progression/pathogenesis in AD and provides a better understanding of Nrf2-mediated modulation in autophagy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

1. Lack of Nrf2 in APP/PS1 mouse exacerbates A β peptide pathology in hippocampus.
2. APP clearance is hindered in Nrf2 deficient APP/PS1 mouse hippocampus.
3. Nrf2-deficient APP/PS1 mouse have altered mTOR pathway in hippocampus.
4. Genetically deleting Nrf2 in APP/PS1 mouse modulated autophagy in hippocampus.

Figure 1. Nrf2-dependent mRNA and protein levels in APP/PS1 mice

Seven-month-old mice were used here and in all subsequent studies. A) Relative levels of mRNA expression in hippocampus. B) Protein levels of Keap1, Nqo1, GCLM and GCLC from 7-month-old APP/PS1 and APP/PS1/Nrf2^{-/-} mice and their respective littermate controls hippocampus. C) Densitometry analysis of Keap1, Nqo1, GCLM and GCLC protein levels from B. Data are presented as fraction (A) and percent (C) of WT values (mean ± SEM; n=3). *Significantly different from the corresponding values for APP/PS1 negative mice (p<0.05); #Significantly different from the corresponding values for Nrf2^{+/+} values (p<0.05).

Figure 2. Increased astrocytic and microglia activation in hippocampus APP1/PS1/Nrf2^{-/-} mice
 A) immunofluorescence staining for GFAP in CA1 region of hippocampus. B) mRNA expression levels of GFAP in hippocampus. C) immunofluorescence staining of IBA1 in CA1 region of hippocampus. D) mRNA expression levels of IBA1 in hippocampus. E) Protein levels of GFAP and IBA1 and F) Densitometry analysis of GFAP and IBA1 in E. Data are presented as percent of WT values (mean $\pm$ SEM; n=3). *Significantly different from the corresponding values for APP/PS1 negative mice (p<0.05); #Significantly different from the corresponding values for Nrf2^{+/+} values (p<0.05).

Figure 3. Increased AD-like Pathology in APP/PS1/Nrf2^{-/-} hippocampus

A) Hippocampus sections were immunohistochemically stained with 6E10 (a and b: 10X magnification; c and d: 20x magnification of CA1 neuronal field) and 4G8 (e and f: 10X magnification; g and h: 20x magnification of CA1 neuronal field). B), 6E10 immunoblot from Triton-X100 soluble and urea soluble fractions.

Figure 4. Increased Aβ (1-40) and Aβ (1-42) in hippocampus of APP/PS1/Nrf2^{-/-} mice
A) Sections were stained with Aβ (1-42)-specific antibody, AB5078P, (a-d) and Aβ (1-40)-specific antibody, AB5074P, (c, d). **B)** Human Aβ (1-42) quantified by ELISA. Data presented as pmol/mg wet weight of tissue. *Significantly different from APP/PS1 mice (p<0.05).

Figure 5. Total APP-FL protein levels do not change but increases in hippocampal neurons
 A) Protein levels of APP-FL and CTFs were detected by using antibodies 22C11, 6E10, C1/6.1 (C-terminal specific). B) Densitometry analysis of the APP-FL (22C11) western blot in A. C) Densitometry analysis of the CTFs (C1/6.1) western blot in A. D) Sections were immunohistochemically stained with 22C11 (a and b: 10X magnification, plaques were immuno-positive for 22C11 staining (arrowheads); c and d: 20x magnification of CA1 neuronal field. Data are presented as percent of WT values (mean $\pm$ SEM; n=3).
 *Significantly different from the corresponding values for APP/PS1 negative mice (p<0.05).

Figure 6. APP-FL is increased in the insoluble fraction of the APP/PS1/Nrf2^{-/-} hippocampus
Short and long exposures of western blots for APP-FL using the 22C11 antibody in the triton-X soluble (A) and urea-soluble fractions (B).

Figure 7. Altered autophagic substrate and autophagy marker in hippocampus of APP/PS1/Nrf2^{-/-} mice.

A) Protein levels of p62 (Sqt1) from total hippocampal lysate, triton-X-soluble fraction and urea-soluble fraction. B) Densitometry analysis of the p62 western blots in A. C) immunofluorescent staining of p62 in the CA1 neuronal field from APP/PS1 and APP/PS1/Nrf2^{-/-} mice. D) Protein levels of LC3BI and II. E) Densitometry analysis of LC3BI and II in D. Data are presented as percent of WT values (mean ± SEM; n=3). *Significantly different from the corresponding values for APP/PS1 negative mice (p<0.05); #Significantly different from the corresponding values for Nrf2^{+/+} mice (p<0.05).

Figure 8. Increased accumulation of poly-ubiquitin conjugated proteins in hippocampus APP/PS1/Nrf2^{-/-} mice

Immunoblot analysis for poly-ubiquitin conjugated protein (VU1 antibody) from Triton-X soluble (A) and urea soluble (B). C), immunofluorescence staining for poly-ubiquitin conjugated protein (VU1, Green) in CA1 neuronal field co-labeled with β-III Tubulin (red). Arrowheads are pointing to poly-ubiquitin conjugated that have accumulated inside the CA1 neurons.

Figure 9. The mTOR pathway is activated in APP/PS1/Nrf2^{-/-} mice

A) Protein levels of mTOR, Phospho-mTOR. B) Densitometry analysis and the ratios of phospho-mTOR to total mTOR. C) Protein levels of p70S6k and phospho-p70S6k. D) Densitometry analysis and the ratios of phospho-p70S6k to total p70S6k from C).

Figure 10. Deletion of Nrf2 gene in APP/PS1 mouse modulates Lysosomal-associated membrane protein and its associated hydrolase protein levels

A), Lysosomal marker Lamp1 co-labeled with 6E10 in CA1 neuronal field from 7-month-old APP/PS1 and APP/PS1/Nrf2^{-/-} mice. D) Protein levels of lysosomal markers Lamp1 and Lamp2a. E) Densitometry analysis of the western blots in D. F) Protein levels of lysosomal hydrolase Cathepsin D (pre- and mature). G) Densitometry analysis of the western blots in F. Data are presented as percent of WT values (mean $\pm$ SEM; n=3).

*Significantly different from the corresponding values for APP/PS1 negative mice (p<0.05);

#Significantly different from the corresponding values for Nrf2^{+/+} mice (p<0.05).

Figure 11. Increased multivesicular bodies, endosomes and lysosomes in APP/PS1 mouse lacking Nrf2 compared to APP/PS1 mice

Lower and higher magnification TEM images for whole cell body of a CA1 neuron in hippocampus from 7-month old APP/PS1 mouse (A and C respectively) and APP/PS1/Nrf2^{-/-} (B and D respectively). Arrows denote the multivesicular bodies, endosomes and lysosomal vacuoles. Examples of dying CA1 neurons in the APP/PS1/Nrf2^{-/-} (E and F). Scale bar: 5 μ m (Panel A, B, E and F) and 2 μ m (Panel C and D).