Published in final edited form as:

Brain Res. 2008 November 19; 1241: 176–187. doi:10.1016/j.brainres.2008.08.086.

TGF β 2-INDUCED CHANGES IN LRP-1/T β R-V AND THE IMPACT ON LYSOSOMAL A β UPTAKE AND NEUROTOXICITY

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Abstract

Numerous studies suggest a central role for the low-density lipoprotein receptor-related protein/transforming growth factor beta receptor V in Alzheimer's Disease. We continue our investigation of a ligand for this receptor, transforming growth factor beta2, which is also implicated in Alzheimer Disease pathogenesis, but whose mechanism(s) remain elusive. Confocal imaging reveals that transforming growth factor beta2 rapidly targets amyloid beta peptide to the lysosomal compartment in cortical neurons and induces cell death. Low-density lipoprotein receptor-related protein/transforming growth factor beta receptor V is known as an endocytic receptor, delivering proteins to the lysosomal compartment for degradation. Transforming growth factor beta2 may alter this pathway resulting in increased uptake, intracellular accumulation and toxicity of amyloid beta peptide. RT-PCR and Western blot analysis of transforming growth factor beta2-treated cells demonstrate that transforming growth factor beta2 modestly increases the mRNA and protein levels of low-density lipoprotein receptor-related protein/transforming growth factor beta receptor V as well as increases the uptake activity. Furthermore, transforming growth factor beta2 alters the morphology and numbers of lysosomes in neurons. Lucifer yellow and lysosomal hydrolase analysis show that transforming growth factor beta2 makes lysosomal membranes unstable and leaky and this effect is exacerbated with the addition of amyloid beta protein. Our data support a key role for low-density lipoprotein receptor-related protein/transforming growth factor beta receptor V in mediating transforming growth factor beta2 enhancement of amyloid beta peptide uptake and neurotoxicity.

Keywords

Alzheimer's Disease; Growth Factors; Amyloid; Lysosome; Cell death

1. INTRODUCTION

The transforming growth factor beta (TGF β) family is comprised of three mammalian isoforms: TGF β 1, - β 2 and - β 3. TGF β s have diverse and important functions throughout the body including growth and differentiation of numerous cell types, immune regulation, induction of extracellular matrix production, tissue repair and putative roles in a number of disease processes

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Senior Editor: Frank R. Sharp (Sacramento, CA, USA)

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including glioblastoma, malaria, multiple sclerosis, progressive supranuclear palsy and Parkinson's and Alzheimer's diseases (AD) [13;15;35;46;48]. Studies have shown that of the three isoforms of TGF β , TGF β 2 may be of highest importance in the pathology of AD [35; 36]. Immunoreactivity of TGF β 2 is increased in AD brain relative to age-matched controls, and both reactive glia and neurofibrillary tangle-bearing neurons display strong TGF β 2 immunoreactivity [15;45]. Risk factors associated with AD, such as inflammation and head injury can lead to elevated levels of TGF β 2 [32;53]. A β 42 has also been shown to induce glial and neuronal TGF β 2 expression [23].

The actions of TGF β are mediated by TGF β receptors (T β R) I through III. T β R-I and R-II are Ser/Thr-specific protein kinases and are thought to be responsible for most TGF β -induced cellular responses [39]. T β R-III is a proteoglycan-containing membrane protein that presents the ligand to other TGF β receptor types and has recently been reported to regulate signaling mediated by the T β R-I/T β R-II heterocomplex [3;4]. T β R-V coexpresses with T β R-I, T β R-II, and T β R-III in most cell types [44] and has recently been shown to be identical to the low density lipoprotein receptor-related protein-1 (LRP1) [24]. It is clear that the different isoforms of TGF β have different cellular functions [25] including different affinities for TGF β receptors [9;39;40].

LRP1/T β R-V is known for being an endocytic receptor but also functions as a signaling receptor [21]. LRP1/T β R-V binds a wide variety of ligands such as alpha-2-macroglobulin, apolipoprotein E, insulin growth factor binding protein-3 and TGF β s 1 and 2, suggestive of an important and central role in the pathogenesis of AD [6;21].

In this study, we sought to determine if TGF β 2 regulates LRP and lysosomal accumulation of A β and what role this may play in A β neurotoxicity.

2. RESULTS

LRP1 is an endocytic receptor thought to endocytose A β and degrade it through the endosomal/lysosomal system. LRP1 is also identical to the Type V TGF β receptor (T β R-V). The TGF β dependent enhancement of A β uptake appeared to require the LRP receptor as murine embryonic fibroblasts that are deficient in LRP (PEA13 cells) did not take up A β like LRP-expressing MEF1 (murine embryonic fibroblasts) cells (Figure 1). MEF1 cells treated with 100 ug/ml A β 42 alone for 3 d showed a small but significant ($p < 0.05$) increase in A β 42 uptake. However, A β 42+TGF β 2 treated MEF1 cells took up much more A β 42 ($p < 0.001$ vs. A β 42 alone). Cells stained with pre-adsorbed 10G4 antibody did not display any A β -ir. MEF1/PEA13 cells possess T β R receptors I and II necessary for traditional TGF β signaling [24]. Despite the presence of traditional TGF β signaling, only the lack of the LRP1 receptor prevented the TGF β 2-mediated uptake of A β .

We sought to determine what effect TGF β 2 had on the expression of LRP1 protein and mRNA and receptor activity. TGF β 2 increased LRP1 mRNA and protein levels (Figure 2). A 3-day treatment of cell cultures with 0.5 ng/ml TGF β 2 resulted in an increase in LRP1 protein levels [Fig 2A: Cortical neurons $*p = 0.05$; Fig. 2B: MEF1 $*p = 0.026$; Fig. 2C: PC12 (rat pheochromocytoma) cells $*p = 0.03$] and LRP1 mRNA (Fig. 2E: Cortical neurons $*p = 0.0001$; Fig. 2F: MEF1 cells $*p = 0.038$; Fig. 2G: PC12 cells $*p = 0.001$). Figure 2D is a representative western blot for LRP1 (5A6 antibody) in control and TGF β 2-treated cortical neurons. Actin staining is also shown to demonstrate equal loading of protein in each lane. Interestingly, PC12 cells are reported not to contain Type II TGF β 2 receptors (necessary to recruit Type I receptors and initiate traditional TGF β signaling pathways) [38], yet TGF β 2 still increased LRP1 mRNA and protein levels suggesting the TGF β 2 effect is not mediated by the classical TGF β signaling pathway but through LRP1/T β R-V. A similar trend for increases in LRP1 protein and mRNA

were seen after 1d of treatment but in some experiments, the trends did not reach statistical significance (data not shown).

In addition to increased LRP mRNA levels and protein following TGF β 2 treatment, we also observed a modest increase in the uptake function of LRP using C14-labeled A2M as the uptake probe (Figure 2H; * p = 0.032). However, despite a 75% or higher increase in LRP1 proteins levels in the three cell types, this did not translate into a similar increase in uptake activity.

Consistent with our previous in vitro and in vivo data, TGF β 2 treatment increased neuronal A β 42 immunoreactivity (ir) [19;20] and increased A β 42ir in the lysosomal compartment (Fig. 3, *B* and *D*; * p = 0.0004). Primary cortical neurons were treated with A β 42 with and without TGF β 2 for 1–7 days and fluorescently stained (red) with a lysosomal antibody to LAMP1 (lysosomal-associated membrane protein 1) or an end-specific A β 42 antibody (green). Figure 3 shows the confocal overlay images of LAMP1 and A β 42 fluorescence. Compared to vehicle-treated neurons (Fig. 3A), A β 42 treated neurons (Fig. 3B) showed a non-significant increase in A β 42 fluorescence that was not correlated with LAMP1 fluorescence. 1d or 3d of A β 42 +TGF β 2 treatment (Fig. 3C and 3D respectively), dramatically increased A β 42 fluorescence and co-localization with LAMP1 fluorescence. Pearson's correlation of fluorophore overlap was significant only for the A β 42 + TGF β 2 treated neurons (Fig. 4A; * p = .0003). Figure 4B shows the treatment effects on the individual correlation coefficients k_1 (for A β 42) and k_2 (for LAMP1). In the control and TGF β 2 treated groups, LAMP1 fluorescence (k_2) contributes mostly to any fluorophore overlap occurring in the image (Fig. 4C). As A β 42 is added to the treatment, the k_1 value starts to increase and approaches the k_2 value in the A β 42+TGF β 2 treated group (Fig. 4D).

The distribution of the co-localization of A β and LAMP1 changed from 1d to 3d (Fig. 3C and 3D, respectively). On day 1, the A β 42/LAMP1 colocalization was found perinuclearly in relatively small lysosomes. By day 3, the antigens co-localized into larger vesicles that had moved out towards the neuron's periphery. By day 7 of treatment, there was no DAPI staining visible in the A β 42+TGF β 2 treated group, and all neurons showed evidence of cell death (not shown). LDH measurements over time revealed that LDH levels started to increase in the A β 42+TGF β 2 treated group at 8 hours and continued to increase over time (data not shown). No significant changes in LDH release occurred in any other treatment group.

TGF β 2 treatment alone (3 d treatment) had a significant impact on LAMP1-ir, causing an increased density of LAMP1-ir vesicles (Fig. 4C; * p = 0.01). Micrographs revealed larger vesicles and a greater portion of the cell stained for LAMP1 (Fig. 3E), consistent with an increased proliferation of vesicles.

LRP-1 binds and internalizes numerous, structurally diverse ligands, delivering most but not all these ligands to lysosomes for degradation. We sought to determine if TGF β 2 could alter the function of the lysosome by itself and enhance A β -mediated lysosomal damage. Lucifer Yellow (LY) is a marker of fluid-phase pinocytosis that accumulates in secondary lysosomes [51] and is impermeable to cellular membranes [2]. Cortical neurons were loaded for 16 hours with LY and then treated for 12 hours (Figure 5A). Control neurons had clear punctuate labeling of lysosomal vesicles. TGF β 2-treated neurons also had abundant LY-positive punctuate vesicles but often the fluorescence was more diffuse throughout the cell body indicating leakage of LY from the secondary lysosome into the cytosol. This trend was not significant at 12 hours but in a separate 24 hour experiment, TGF β 2 treatment alone caused a significant 40% increase in LY fluorescence (p < 0.001 versus control; data not shown). A β alone also caused a small increase in LY fluorescence after 12 hours of treatment but combined with TGF β 2, the increase in lysosomal leakiness was dramatic. In A β +TGF β 2 treated neurons, LY

fluorescence nearly completely filled the cell body and extended into the cell processes. LY fluorescence was quantitated as area (in microns) and is shown in Figure 5B.

We next sought to determine if TGF β 2 treatment altered the primary endosomal compartment. A significant fraction of lysosomal hydrolases reside in the primary endosomal compartment. The leakage of β N-Acetylglucosaminidase (NAG) into the cytosol was determined by subcellular fractionation of cortical neurons treated for 24 hours with TGF β 2 (Figure 5C). The amount of soluble NAG activity present in the cytosolic fraction (cytosolic NAG activity expressed as a percentage of total cellular NAG activity) was not significantly higher in any treatment group indicating no alteration in the fragility of the primary lysosomal membrane.

3. DISCUSSION

This study supports a role for TGF β 2 in altering LRP and lysosomal levels and activity and facilitating intracellular A β accumulation and neurotoxicity. Supporting our previous work [19;20], co-treatment with TGF β 2 rapidly increased intracellular accumulation of A β 42 and caused cell death. A β is known to be present in intracellular compartments, intracellular accumulation of A β appears early in AD pathology [27;49] and A β 42 first accumulates in the perikaryon of pyramidal neurons as discrete granules that are cathepsin D (lysosome) positive [10;11]. We have previously demonstrated in vivo A β accumulation in lysosomal compartments in a rat model of AD [16], alterations in the endocytic pathway are a very early response in AD [7] and the failure to degrade aggregated A β 42 in late endosomes or secondary lysosomes may account for the accumulation of A β in AD [17]. The reason for this failure of A β degradation and clearance is not known but our studies suggest that TGF β 2 may impair A β clearance from neurons. The breakdown of the lysosomal membrane may be a key pathogenic event leading to the release of heparan sulfate and lysosomal hydrolases into the cytosol and resulting in increased susceptibility of neurons to injury, amyloidogenesis and neurodegeneration [43]. The loss of lysosomal membrane impermeability is correlated with A β 42 accumulation [57].

Neuronal cell death in AD has been shown to correlate with ApoE uptake and intracellular A β stabilization [31]. LRP appears to be the common link among multiple proteins relevant to AD. Three of the genes where mutations impart increased susceptibility to AD: APP, ApoE and A2M, encode proteins that are ligands for LRP [1;28–30;42;47;50]. A β binds avidly to LRP receptor ligands such as A2M and ApoE, and LRP-mediated clearance of A β can lead to A β in the lysosome. Though TGF β 2, also an LRP ligand, maintains a low level of expression in control brain, expression is increased in AD brain [8;15;34;56] and is localized in plaque neurites and neurofibrillary tangle (NFT)-bearing neurons which correlate [15] well with neuron loss [45]. TGF β exacerbation of A β neurotoxicity has been previously reported to occur in the absence of “classical” type I/II TGF β receptors [41] although this does not exclude a role for this pathway in regulating LRP activity. Recent studies have revealed a potential link between LRP and TGF β s [24].

When considering the role of LRP on clearance of A β , it is important to distinguish between LRP at the blood brain barrier (BBB) and LRP on neurons. Transport of A β from the bloodstream into the brain is mediated by RAGE (receptor for advanced glycosylated end products) [12;14], and A β transport from the brain into the bloodstream is mediated by LRP1 [18;28;29;52;54]. The net flux of A β into or out of the brain is the algebraic sum of inward and outward flux and presumably depends upon the density and activity of these two receptors. Numerous studies have shown not only that LRP1-ir on endothelial cells is reduced in AD, but there also appears to be an increase in endothelial RAGE-ir [14]. Taken together, the result is a net increase in A β transport into the interstitial space of the brain in AD. This added A β is likely to be in excess of what can be removed via CSF turnover and clearance, which is also

reduced in AD. Donahue et al., [14] have demonstrated that although LRP1 is decreased at the BBB in AD brain, LRP1 is actually increased on neurons in the AD brain. Increased LRP1 on neurons could be a reactive response of the brain to increased A β levels and it has been suggested that increased LRP levels are protective because of increased A β clearance [55]. However, under pathologic conditions where TGF β 2 is driving changes in LRP1 and endocytic pathways, our current data suggest this condition will not be protective but neurotoxic. Shifts from protection to toxicity may involve alterations in location of the LRP1 or differential activation of cell signaling pathways.

A pathogenic role for LRP1 is also supported by data from an APP and LRP minigene overexpressing transgenic mouse model, showing an increase in the pool of small, soluble form of A β that correlates with impairments in spatial learning & memory [60]. In these same LRP mini-gene/APP mice, A β 42 frequently co-localized with LAMP-1 at a time point prior to amyloid plaque development [59] demonstrating a link between increased LRP levels and lysosomal accumulation of A β . Increased soluble A β could occur if there was increased uptake of A β or increased A β production. In addition to uptake of exogenous A β , LRP1 has a pivotal role in the trafficking of APP and the generation of A β [33] and recent work demonstrates that gamma-secretase cleavage of APP plays a central role in regulating apoE and cholesterol metabolism in the CNS via LRP1 [37]. Interestingly, TGF β 2 is a binding protein for soluble APP derivatives [5], suggesting a mechanistic link between APP and TGF β 2 in the disease process, that may result from the impact of LRP1 on APP processing. Studies are ongoing to determine how TGF β 2 may alter APP processing and what role, if any, APP and APP signaling may play in our observed TGF β 2-induced A β 42 lysosomal accumulation and toxicity. Specific alterations in cell signaling pathways are likely involved as the binding of TGF β 2 to APP activates an APP-mediated death pathway involving heterotrimeric G protein Go, c-Jun N-terminal kinase, NADPH oxidase, and caspases [22]. Our data showing a very modest increase in uptake activity despite a 75% or higher increase in LRP1 proteins levels would suggest that TGF β 2 may function primarily by altering cell signaling activities.

It is not yet clear what increases TGF β 2 levels in the AD brain. Risk factors for AD such as inflammation and oxidative stress may change TGF β 2 levels. Furthermore, the outcome of these changes in TGF β 2 levels can depend on the activation state of the cell. In AD, TGF β 2 may drive pathology under conditions of altered APP processing, A β , ApoE or other co-factors. The role that the different isoforms of ApoE play in the observed TGF β 2 effects is currently under investigation. We have shown that TGF β 2 can rapidly alter LRP expression and activity. In addition, TGF β 2 causes a proliferation of lysosomal vesicles where A β 42 accumulates and causes cell death. Instability in lysosomal membranes in the presence of TGF β 2 and/or A β 42 may lead to activation of cell death pathways and the demise of the cell. Future studies may also examine A β 40 and A β 42/A β 40 ratios on TGF β 2-mediated A β uptake into lysosomes.

4. EXPERIMENTAL PROCEDURES

A β Preparation and Treatment

Oligomer was prepared as previously described [26]. Briefly after monomerizing lyophilized A β with hexafluoroisopropanol (HFIP), 4 mM HEPES buffer (pH 7.0) was added to prevent fibrils forming from rapid HFIP evaporation. After evaporation of HFIP, the A β solution (300 ug/ml) was incubated for 24-hr at 4°C to enhance aggregation of monomer, A β 42 was diluted in culture media (10 ug/ml for MEF1 and PEA13 fibroblasts and 2.5 ug/ml for cortical neuron). A β was obtained from C. Glabe (University of California, Irvine) and TGF β 2 from R & D Systems (Minneapolis, MN). LDH dose-response curves were generated for a wide range of TGF β 2 concentrations to determine a concentration that would not induce toxicity even after 7 days of treatment. The dose of TGF β 2 used in all experiments was 0.5 ng/ml.

Cell Culture

Use of animals was approved by the VA-Greater Los Angeles Area Healthcare System Institutional Animal Care and Use Committee. Primary cultures of cortical neurons were prepared as described previously [61]. Briefly, cortices were dissected from the brains of embryonic day 17 (E17) C57 mouse fetuses, treated with 0.05% trypsin in HBSS without Ca^{2+} or Mg^{2+} for 5 min at 37°C, and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. For immunocytochemical staining analyses, cells were seeded onto poly-D-lysine (10 $\mu\text{g}/\text{ml}$)-coated glass coverslips in 24-well plates, and cultures used for Western immunoblotting and lactate dehydrogenase (LDH) analyses were plated onto poly-D-lysine-coated 6-well plates at a density of 1 million neurons per well. Nerve cells were grown in Neurobasal medium (NBM; Invitrogen, Carlsbad, CA) supplemented with B27, 5 U/ml penicillin, 5 $\mu\text{g}/\text{ml}$ streptomycin, 0.5mM glutamine, and 25 μM glutamate, at 37°C in 10% CO_2 . Cultures grown in serum-free NBM yielded $\geq 99.5\%$ neurons. Microscopically, glial cells were not apparent in hippocampal cultures at the time they were used for experimental analyses. The culture media was exchanged with glutamate-free NBM 3d after the day of cell culture. Experiments were conducted on cortical neurons that were 7–10 d old.

Rat pheochromocytoma PC12 cells (ATCC: CRL-1721) were cultured on rat tail collagen-coated plates. Both PC12 cells and human neuroblastoma SH-SY5Y cells (ATCC: CRL-2266) were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum (PC12) and 5% fetal bovine serum at 37 °C (5% CO_2). Cells were differentiated to a neuronal phenotype using 100 ng/ml of NGF.

LRP-1-deficient fibroblasts (referred to interchangeably as MEF-2 or PEA-13 cells in the literature) and LRP-1-expressing MEF-1 cells were obtained from ATCC and cultured in high glucose DME with 10% FBS and penicillin/streptomycin. At the end of A β treatment incubation, the cells were washed twice with PBS and incubated with 500 $\mu\text{g}/\text{ml}$ trypsin-EDTA at 4°C for 15 min to remove any nonspecific, cell surface-associated A β .

Immunocytochemistry and Image Analysis

After treatment, neurons on coverslips were washed briefly with PBS and then fixed with 50% HPLC-grade methanol/50% Acetone at 4°C. Nonspecific antibody binding is blocked by incubation in blocking buffer (0.3% Triton-X100, 3% BSA in PBS) for 45 min at room temperature. Coverslips were then incubated with primary antibodies against A 42 (rabbit polyclonal, 1:50; Biosource) and LAMP-1 (rat polyclonal 1D4B, 1:50; Univ. of IA). All primary antibodies were dissolved in PBS. Alexa Fluor568 goat anti-rat (Molecular Probes A11006) and Alexa Fluor488 goat anti-rabbit (Molecular Probes A11011) were used at a dilution of 1:200. Secondary antibodies dilutions were prepared in 3% BSA with 5% Normal Goat Serum (NGS). Sections were incubated, in the dark, for 1 hour at room temperature. Coverslips were washed extensively and coverslipped with 4-,6--diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories) and images captured using a 2-photon laser scanning confocal microscope system in the Carol Moss Spivak Cell Imaging Facility at the University of California, Los Angeles (http://www.gonda.ucla.edu/bri_core/homepage.htm). A Leica TCS-SP confocal inverted microscope equipped with argon, krypton, and HeNe lasers provides wavelengths of 488, 568, and 633 nm. Images were captured using a 63x oil immersion objective.

For A β immunohistochemistry of MEF1 and PEA13 fibroblasts (10G4 antibody against A β 5–13) [58], cells on glass slides were pretreated with 70% trichloroacetic acid (6 min) and rinsed with TBS. Endogenous peroxidase activity was suppressed using a peroxidase suppressor buffer (Vector Labs) for 30 min at room temperature and nonspecific binding sites were blocked with Superblock Blocking Buffer (Pierce, Rockford, IL) for 1 hr at room

temperature. Anti-A β was applied (overnight at 4°C) to slides at a 1:500 dilution in TBS containing 0.1% Tween 20, 3% bovine serum albumin (BSA), and 8 mM sodium azide. Slides were then rinsed and incubated with a biotinylated anti-mouse secondary antibody (1:500 in TBS plus Tween 20; 1 hr at room temperature). Slides were rinsed and incubated with the avidin-biotin peroxidase complex (Elite ABC kit; Vector Labs) for 45 min at room temperature, and developed with diaminobenzidine-metal enhanced chromogen (Pierce). The specificity of A β staining was verified by preincubating 10G4 antibody in the presence of A β 1–40 peptide before immunostaining. Preabsorption of 10G4 antibody was carried out by adding 1 μ L of antibody to 50 μ L of 3% BSA in TBS containing 30 μ g A β 40 peptide. The solution was incubated overnight at 4°C, brought up to 800 μ L with 3% BSA in TBS, and centrifuged at $14,000 \times g$ for 30 min at 4°C. The resulting supernatant was used for immunocytochemistry.

Image Pro image analysis software was used to quantify immunocytochemically stained cells. For fluorescence images, Pearson's correlation coefficient and overlap coefficients k1 and k2 were used to examine co-localization of fluorophores. Pearson's correlation coefficient was one of the standard measures in pattern recognition. It was used for describing the correlation of the intensity distributions between channels. Overlap coefficients k1 and k2 split the value of colocalization into the two separate parameters. These coefficients reflected the contribution of each channel to the process of colocalization. In our experimental conditions, coefficient k1 indicated a contribution of A β 42 (green), while coefficient k2 showed a contribution of LAMP-1 protein (red). Data were exported into spreadsheet files for statistical analysis. Images of immunocytochemically stained cells were assembled into montages using Adobe Photoshop 7.

Western blot

Protein concentration was determined by the Bio-Rad (Hercules, CA) DC protein assay. Except for A Western detection, samples (50–75 μ g of proteins per well) were boiled at 100°C for 4–5 min, loaded on 4–12% Tris-glycine gradient gels from Invitrogen (Cat.# EC6038BOX) and resolved by standard electrophoresis at constant 125V and max. 500mA. Proteins were then electrophoretically transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes from Millipore (Cat.# ISEQ00010) for 2 hr at constant 90V and max. 500mA. For A species separation, the protein samples were resolved on 10–20% Tris-Tricine gradient gels at constant 125V and max. 500 mA and transferred to Immobilon-P PVDF membranes for 1.5 hr at constant 90V and max. 500mA. Membranes were blocked for 1 hr at room temperature in 10% nonfat dried milk in PBS and then incubated overnight at 4°C with appropriate primary antibodies (LRP 85 kDa fragment: 5A6 antibody, Molecular Innovations, Inc., Novi, MI) in PBS containing 0.05% Tween 20 (PBS-T) and 5% nonfat dried milk. The membranes were rinsed with PBS-T and incubated with horseradish peroxidase-conjugated anti-mouse IgG from Bio-Rad (Cat.# 170–6516) (1:5,000 to 1:10,000) or anti-rabbit IgG from Pierce (Cat.# 31463) (1:30,000 to 1:100,000) in PBS-T with 5% nonfat dried milk for 1 hr. Immunoreactive bands were visualized by ECL Western blotting detection reagents from Amersham (Cat.# RPN2209), SuperSignal West Dura fExtended Duration Substrate from Pierce (Cat.# 34076) or SuperSignal West Femto Maximum Sensitivity Substrate from Pierce (Cat.# 34096) and quantified using densitometric software (Molecular Analyst II, Bio-Rad, Richmond, CA). After transfer, gels were stained with Coomassie blue to ensure equal protein loading. For LRP Western detection, Broad Range Prestained Protein Marker (6–175 kDa) from BioLabs (Cat.# P7708S) was used. For A Western detection, Low-Range Rainbow Molecular Weight Markers (2.5–45 kDa) and Recombinant Protein Molecular Weight Markers (10–250 kDa) from Amersham (Cat.# RPN755 & RPN800) were used.

Real Time Quantitative PCR Analysis of LRP1 Gene Expression

After scraping of the cells, the samples were stored in an RNA-protective commercial buffer (RNAlater (Ambion Inc., Austin, Texas) at -20°C . Total RNA was extracted using TRI reagent (Sigma, St. Louis, Missouri) and BCP (MRC, Cincinnati, Ohio) as a phase separation reagent and quantified spectrophotometrically. RNA ($1\mu\text{g}$) was reversely transcribed to complementary DNA (cDNA) using an Omniscript RT kit (Qiagen, Valencia, California). Real Time Quantitative PCR, was performed for quantification of mouse low density lipoprotein receptor-related protein 1 (LRP1) mRNA using the ABI 7300 Sequence Detection System® (Applied Biosystems, Foster City, California). PCR amplifications of LRP1 (Assay ID Mm00464808_m1) and glyceraldehyde-3-phosphate dehydrogenase (GADPH; Assay ID Mm99999915_g1) genes were carried out with the Universal PCR Master Mix (ABI) using TaqMan Gene expression (Applied Biosystems). Levels of LRP1 mRNA were normalized to the housekeeping gene GAPDH to correct for possible differences in the amount of each cDNA template. Each cDNA sample was tested in triplicate. The following temperature parameters were cycled for 40 times: 15 seconds at 95°C and 1 minute at 60°C .

LRP activity assay using C-14 alpha-2-macroglobulin

Alpha-2 macroglobulin was radiolabeled with C-14 as follows. Equal amounts of Glutaraldehyde (Sigma Chemical Co., St. Louis, MO, USA) and 14-C methylamine hydrochloride (GE Healthcare, IL, USA) were incubated in PBS for one hour at room temperature before adding alpha 2-macroglobulin (A2M, 1 mg), and subsequently sodium cyanoborohydride (Sigma Chemical Co., St. Louis, MO, USA) for reductive amination. The complex then was washed by 50 mM phosphate buffer (pH 8, containing EDTA) using Nanosep 10K centrifugal filter and centrifuged eight times at $5000\times g$ for four minutes. A2M was activated by 200 mM methylamine-HCl (Sigma Chemical Co., St. Louis, MO, USA) in 50 mM Tris-HCl, pH 8.0, for 2 hours at room temperature. Excess methylamine was removed by dialysis against PBS overnight, and the protein concentration quantified using the bicinchoninic acid (BCA) method. LRP activity was then assessed by measuring C14-A2M uptake by primary cortical neurons as follows. Cells were incubated with 40 ng G-14C-MA-A2M * in 1 ml. media for 1.5–4 h at 4°C (binding) or 37°C (uptake) in 5% CO_2 , and then the monolayers were washed first with Neurobasal Media and then with PBS. The cells were solubilized in 1 ml/well of 0.1 M sodium hydroxide, and cell-associated radioactivity was determined by scintillation counting of washed and solubilized cells.

Subcellular Fractionation Analysis and Determination of β -N-Acetylglucosaminidase (NAG) Activity

The activity of the lysosomal enzyme β N-Acetylglucosaminidase (NAG, N-acetyl- β -D-glucosaminidase), was evaluated by addition of the NAG substrate, 4-Nitrophenyl N-acetyl-b-D-glucosaminide (NP-GlcNAc), and measurement of its hydrolysis to *p*-nitrophenol at 405 nm (Sigma, St. Louis, MO; Product Code CS0780). After incubation with TGF β 2- cells were rinsed extensively with ice-cold PBS and 500 μl homogenization buffer (0.25 M sucrose in 10 mM HEPES, pH7.4, and 1 mM EDTA, 1 mM EGTA, 1 mg/L leupeptin, aprotinin, pepstatin, and antipain) were added to each well. Cells were then removed by a rubber policeman and homogenized with a 2-ml Kontes dounce homogenizer using the B pestle for four strokes. Cell homogenates were pooled and centrifuged at $100,000\times g$ for 30 min. The supernatant was collected as the cytosolic fraction and cell pellet was dissolved in 100 μl homogenization buffer as the lysosomal/mitochondrial fraction. Protein concentration was determined by Bio-Rad (Hercules, CA) DC protein assay. The cytosolic NAG activity (*p*-nitrophenol absorbance/mg protein) was expressed as the percentage of total activity obtained from total cell homogenates (cytosolic plus lysosomal/mitochondrial fractions) from corresponding treatments.

Lucifer Yellow Analysis

Primary cortical neurons were preincubated with 100 mg/ml Lucifer Yellow (LY) in modified DMEM without phenol red (Irvine Scientific, Santa Ana, CA) overnight and were washed at least five times in phosphate-buffered saline (PBS). The Lucifer Yellow labeled cells were then treated with TGF β 2, A β or A β + TGF β 2 in serum-free medium and incubated at 37°C until observation. The coverslips were then rinsed with warm PBS and inverted for viewing on a glass microscope slide with a drop of PBS. The cells were then examined using a 40x objective on a Nikon Eclipse E-400 fluorescent microscope equipped with a SPOT digital camera, IBM PC computer and Image Pro software. Each individual neuron cell body was outlined and the area occupied by Lucifer Yellow was calculated using ImagePro software. 44–66 individual neurons from 12–16 separate glass coverslips were analyzed for each treatment group.

Statistical Analysis

Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test or unpaired t-test using GraphPad Prism 4 statistical software.

Acknowledgements

This work was supported by a National Institutes of Health grant (AG022080) and a Veterans Administration Merit Award to MEH-W. The authors would like to thank D. Holtzman for technical assistance with the Western blot analysis and M. Nyby for assistance with the RT-PCR analysis.

Abbreviations

A2M	Alpha 2 macroglobulin
Aβ	Amyloid beta protein
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
BBB	Blood brain barrier
ir	immunoreactivity
LAMP1	Lysosomal-Associated Membrane Protein 1
LRP	Low density lipoprotein receptor-related protein-1
LY	Lucifer Yellow
MEF	

	Murine embryonic fibroblast
NAG	β N-Acetylglucosaminidase
NFT	Neurofibrillary tangle
PEA13	Murine embryonic fibroblasts that are deficient in LRP
PC12	Rat Pheochromocytoma Cell
RAGE	Receptor for Advanced Glycosylated End Products
TGFβ	Transforming growth factor beta
TβR	Transforming growth factor beta receptors

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Figure 1. LRP knockout cells do not have increased intracellular Aβ
MEF1 (LRP +/+) and PEA13 (LRP -/-) fibroblasts were treated with Aβ42 with or without TGFβ2. Trypsin resistant Aβ-ir area (10G4 antibody) was quantitated and showed a small but significant increase in Aβ-ir with Aβ42 treatment alone (* $p < 0.05$ vs. Control). However, Aβ+TGFβ2 increased intracellular Aβ-ir above Aβ42 treatment alone (** $p < 0.001$ vs. Aβ alone). PEA13 cells did not show increased Aβ-ir in any treatment group. Data are expressed as means $\pm$ SEM of 6–8 separate imaging fields from 6–8 different glass slides.

Figure 2. TGFβ2 increases LRP1 mRNA and protein levels

A 3-day treatment of cell cultures with 0.5 ng/ml TGFβ2 resulted in an increase in LRP1 protein levels in (A) Cortical neurons $*p = 0.05$, (B) MEF = 0.026, (C) PC12 cells $*p = 0.03$ and mRNA in (E) Cortical neurons $*p = 0.0001$, (F) MEF cells $*p = 0.038$, and (G) PC12 cells $*p = 0.001$. (D) Representative western blot for LRP1 in control and TGFβ2-treated cortical neurons. (H) A modest increase in the uptake activity of LRP was observed using C14-labeled alpha-2-macroglobulin as the probe ($*p = 0.032$). Data are expressed as means $\pm$ SEM of 3–8 samples from two separate experiments.

Figure 3. TGFβ2 increases lysosomal accumulation of Aβ42

Cortical neurons were treated for 1–3 days and immunofluorescently labeled to detect Aβ42 (green) and LAMP1 (red). Panels A–D are overlay images showing colocalization of Aβ42 and LAMP1. Compared to Control (A) and Aβ42 treatment alone (B), Aβ42+TGFβ2 treated neurons showed accumulation of Aβ42 within lysosomes 1 d (C) and 3 d (D) after treatment. The distribution of the colocalization in the Aβ42+TGFβ2 treated neurons changed from 1 d to 3 d. After 1 d, the colocalization was distinctly perinuclear and shifted to larger vesicles after 3 d that tended to be located more peripherally in the neuron. Panel (E) is a single fluorophore image of LAMP1-ir in a neuron treated with TGFβ2 for 3d. Compared to control, TGFβ2 increased the percentage cell area stained for LAMP1-ir, suggesting a proliferation of

LAMP1-ir vesicles. Images were captured on a Leica confocal microscope system using a 63x oil immersion objective.

Figure 4. Quantitation of Aβ42 and LAMP1 colocalization in cultured cortical neurons

(A) Pearson's correlation coefficient is used for describing the correlation of the intensity distributions between channels. Colocalization of Aβ42 and LAMP1 antigens is low in control, TGFβ2 and Aβ42 treated groups but dramatically increases in the Aβ42+TGFβ2 treated neurons (* $p = 0.0003$). (B) The overlap coefficients k_1 and k_2 split the value of colocalization into the two separate parameters and reflect the contribution of each channel to the process of colocalization. When colocalization occurs in control or TGFβ2 treated neurons, k_2 (LAMP1, red) predominates yielding a low k_1/k_2 ratio and reflecting the very low levels of Aβ42 present. In Aβ42 or Aβ42 + TGFβ2 treated neurons, values for k_1 (Aβ42, green) and k_2 are similar, increasing the k_1/k_2 ratio towards the value 1.0 and reflecting their colocalization. Data are expressed as means $\pm$ SEM of 4 separate imaging fields from 3 separate coverslips. (C) LAMP1-ir significantly increases with TGFβ2 treatment (* $p = 0.013$). (D) Intracellular Aβ42-ir increases with Aβ42+TGFβ2 treatment but not with Aβ42 treatment alone (* $p < 0.01$). Data are expressed as means $\pm$ SEM of 15–27 separate cells taken from 12 different coverslips.

Figure 5. TGFβ2 alters lysosomal permeability

Cortical neurons were loaded with Lucifer yellow and then treated with TGFβ2, Aβ or Aβ/TGFβ2 for 12 hrs. (A) Control cells displayed clean, punctuate staining of lysosomal vesicles around the nucleus. TGFβ2-treated neurons displayed a more diffuse pattern of LY fluorescence that often filled the cell body. Aβ/TGFβ2 treated neurons demonstrated LY fluorescence that often filled the cell body and extended in axons and dendrites. Magnification 40x, Bar = 20 μm. (B) LY was quantitated and results are expressed as area in μm² (mean ± SEM of 46–66 separate cells from 12–16 coverslips). (C) Treatment did not cause a significant redistribution of the lysosomal hydrolase, β-N-Acetylglucosaminidase (NAG, N-acetyl-β-D-glucosaminidase) from the lysosome into the intracellular space. NAG activity (p-nitrophenol

absorbance/mg protein) was expressed as the percentage of total activity obtained from total cell homogenates from corresponding treatments. Enzyme activity was expressed as umoles p-nitrophenol/mg protein and are means $\pm$ SEM of 6 separate samples from 2 different experiments.