LRRTM3 interacts with APP and BACE1, influences APP processing and has variants associating with late-onset Alzheimer's disease (LOAD).

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Abstract

Leucine rich repeat transmembrane protein 3 (LRRTM3), exclusively expressed in the central nervous system (CNS), is a member of a novel family of proteins implicated in synaptogenesis. *LRRTM3* resides at the peak of the chromosome 10q linkage region identified for late-onset Alzheimer's disease (LOAD) risk and plasma amyloid β (Aβ) levels. In-vitro knock-down of LRRTM3 was previously shown to decrease secreted amyloid β (Aβ), although the mechanism of this is unclear. Using an in-vitro siRNA knock-down approach in SHSY-5Y neuroblastoma cells overexpressing wild type amyloid precursor protein (APP), we first demonstrated decreased secreted Aβ and sAPPβ, but not sAPPα or APP. We postulate that LRRTM3 influences APP processing via β-site cleaving enzyme 1 (BACE1) given its effects on β-secretase cleavage products. In SHSY-5Y cells overexpressing LRRTM3 with APP and BACE1, we determined that LRRTM3 co-localizes with both proteins in early endosomes, where BACE1 processing of APP occurs. Furthermore, in primary neuronal cultures from Tg2576 mice, overexpressing a mutant form of APP and transduced with adeno-associated virus expressing LRRTM3, APP and LRRTM3 co-localize in the neuronal cell body and processes. In HEK293T human embryonic kidney cells transfected with LRRTM3, this protein co-immunoprecipitates with both endogenous APP and overexpressed BACE1. We genotyped 69 single nucleotide polymorphisms (SNPs) in 1,567 LOADs and 2,082 controls and identified 8 SNPs residing in a haplotype block encompassing 5'UTR-Intron 1 of *LRRTM3*, the multilocus genotypes of which suggestively associated with LOAD risk (p=0.06). These SNPs were genotyped in an independent series of 1,258 LOADs and 718 controls and significant global association with MLGs were found in the combined dataset (p=0.02-0.05). Collectively, these results implicate LRRTM3 in LOAD risk with the potential mechanism of promoting BACE1 processing of APP.

Aims

1) To characterize the intracellular and biochemical interactions between APP, BACE1 and LRRTM3, given the potential functional role of LRRTM3 in promoting BACE1 processing of APP.
2) To accomplish fine-mapping of the *LRRTM3* locus by analyzing 69 SNPs in this region in a LOAD case-control cohort (1,567 LOADs and 2,082 controls) and by follow-up of the significant results in a second independent series (1,258 LOADs and 718 controls).

Methods

***LRRTM3 siRNA, ELISA's and Western Blots*:** SH-SY5Y human neuroblastoma cells were stably transfected with human wild-type APP construct (SH-SY5Y-APP695wt). SH-SY5Y cells were split and transfected with 50 pmols of either si-LRRTM3 (si-LRRTM3; SASI_Hs01_00163676) or si-control (si-control; MISSION®siRNA) with four separate transfections per treatment group. After 24 hours, cells and media were harvested for Western blot analysis, Aβ40, Aβ42, sAPPα and sAPPβ ELISA experiments (see handout for details).

Immunocytochemistry: SH-SY5Y-APP695wt monoclonal cell line, transfected using a full length human LRRTM3 construct with a V5 tag, with or without co-transfection with a human full length BACE1 construct with an influenza hemagglutinin (HA) tag, both expressed within a pcDNA3 vector. Four hours post-transfection, media was removed and replaced with fresh media containing 2µl/ml baculovirus BacMam 2.0 (Invitrogen) expressing a GFP-tagged protein specific for one of the following organelles: Early Endosomes-GFP, Golgi-GFP or Lysosomes-GFP. Nuclei were stained with DAPI (Hoechst H3569 Molecular Probes). Neuronal cultures (Tg2576: APPK670N,M671L) from P2 mice were incubated with primary antibodies V5 (Sigma; 1:1000) to detect LRRTM3 and CT20 (gift from Pritam Das, 1:250) to detect APP and then incubated with green Alexa Fluor 488 goat anti-mouse and red Alexa Fluor 594 goat anti-rabbit secondary antibodies to visualize LRRTM3 and APP, respectively. All images were acquired with the Zeiss LSM510 Laser Scanning Meta confocal microscope.

Co-immunoprecipitation (co-IP): Cultured human embryonic kidney HEK293T cells (70% confluent) were transfected with the indicated expression constructs. Harvested cells were lysed in IP buffer and total protein was determined. IP protocols were followed per the manufacturers instructions (Immunoprecipitaion Kit-Dynabeads®Protein G (100.07D Invitrogen)) Eluted immunocomplexes were resolved on a 4-12% NuPage Bis-Tris gel (Invitrogen) and Western blot assays performed with the indicated primary antibody and appropriate HRP conjugated secondary antibody.

Genetic association studies: Unrelated subjects from six independent LOAD case–control series, consisting of Caucasians with an age-at-diagnosis (LOAD), evaluation (elderly controls) or death (autopsy series) ≥60 years, were utilized in this study (3,166 LOAD vs. 3,261 controls). Sixty-nine SNPs were genotyped using either the Sequenom platform (Sequenom Inc., San Diego, CA, USA) or TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA). Statistical analysis was carried out using the functionalities in the following statistical packages: PLINK (single SNP analysis), Haploview and Haplostats (Haplotype analysis) and StatsDirect (MLG analysis). All analysis included covariates for Age, Sex, APOE4 dosage and Series where appropriate.

Figure 1

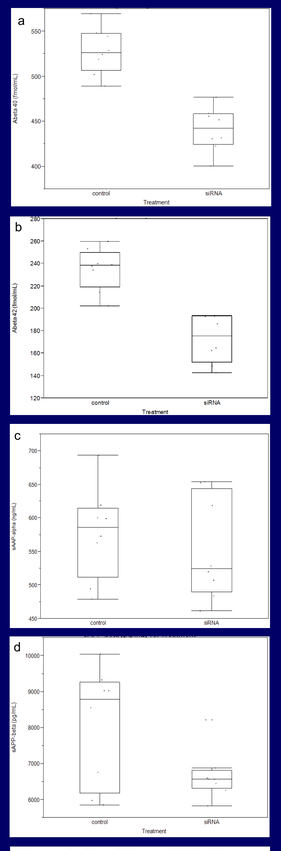

Figure 1. Box Plots of ELISA results: a) Aβ40, b) Aβ42, c) sAPPα, d) sAPPβ levels measured in the media from SH-SY5Y-APP695wt cells transfected with either si-LRRTM3 or si-control (siRNA or control, respectively on x-axis). Levels of Aβ40, Aβ42, sAPPβ are significantly lower in the si-LRRTM3 treated group vs. si-control group, whereas no difference is noted for sAPPα or APP levels.

Figures 2, 3 & 4

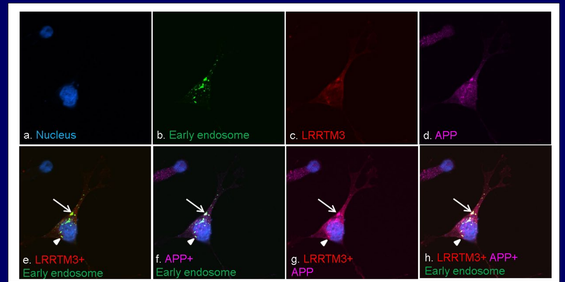

Figure 2. Co-localization of LRRTM3 and APP in early endosomes: Co-localization of APP, LRRTM3 and early endosomes is visualized as white punctate intracellular structures in h (arrow and arrowhead) and can also be seen in e-g. Magnification: x 63

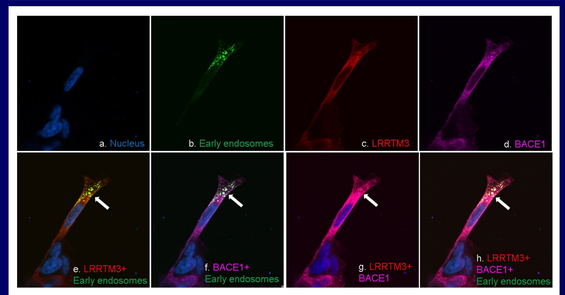

Figure 3. Co-localization of LRRTM3 and BACE1 in early endosomes: Co-localization of APP, BACE1 and early endosomes is visualized as white punctate intracellular structures in h (block arrow) and can also be seen in e-g. Magnification: x 63.

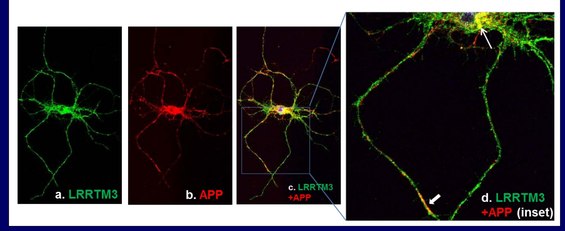

Figure 4. Co-localization of LRRTM3 and APP in primary neurons: Primary neuronal cultures from Tg2576 transgenic mice transduced with rAAV-LRRTM3-V5. Co-localization of LRRTM3 and APP is visualized as yellow puncta in the cell body (thin arrow) and neuronal process (thick arrow). Magnification: x 100.

Figure 5

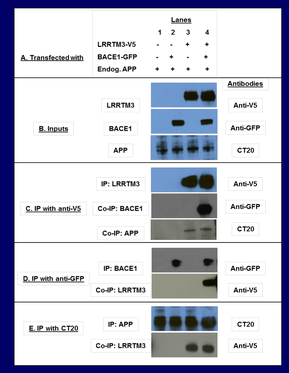

Figure 5. Co-IP of BACE1 and endogenous APP with LRRTM3 in HEK293T cells: LRRTM3-V5 and BACE1-GFP were transfected into HEK293T cells as shown in (a) and as in inputs (b). Protein lysates from these cells and negative controls without overexpression were immunoprecipitated using c. anti-V5 (LRRTM3), d. anti-GFP (BACE1) or e. CT20 (APP) antibodies. Presence (+) or absence (-) of each of the three proteins for the depicted experiments are shown. Antibodies used in the Western blots are listed to the right of each figure. Proteins that are IP'ed or co-IP'ed are shown to the left of each figure.

Table 1

Solid Spine of LD Blocks	#SNPS in block	P-value: Haplotypes	P-value: MLG (cohort1)	P-value: MLG (cohort 2)	P-value: MLG (cohort 1 +2)
1	8	0.94	**0.059**	0.55	**0.036**
2	2	0.72	0.92	na	na
3	10	0.80	0.44	na	na
4	6	0.98	1.00	na	na
5	2	na	na	na	na
6	23	0.16	0.94	na	na
7	18	0.47	0.97	na	na

Table 1. Significance of haplotype and MLG associations: In cohort 1, haplotype blocks were determined by solid spine of LD within Haploview. Haplotype associations for each block was tested within HaploStats, using an additive model and controlling for the age, sex, APOE4 dose and series effects. MLG analysis for SNPs within each block was likewise done with logistic regression analysis while controlling for the same covariates.

Conclusions

- Our findings demonstrate intracellular and biochemical interactions between LRRTM3 and APP and BACE1 for the first time.
- We identify a region in the 5'UTR-Intron 1 of LRRTM3 that has variants which associate with LOAD risk.

These results have implications for future genetic and functional studies of this gene and its role in AD.