<u>SUPPLEMENTARY MATERIALS</u> (Malkus et al.)

SUPPLEMENTARY EXERIMENTAL PROCEDURES

Immunohistochemistry. Perfusions, tissue processing, and staining were performed as described previously (Giasson et al., 2002). Briefly, 6 µm coronal sections from the brains of mice perfused with 70% EtOH and embedded in paraffin and stained with an antibody specific for human α-syn, Syn303 (Duda et al., 2002). Incubation with secondary antibodies conjugated to biotin was followed by addition of avidin-biotin peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, CA), visualized with 3,3'-diaminobenzidine as the chromogen, and counterstained with hematoxylin (SH30-500D; Thermo Fisher Scientific, Rockford, IL).

Western Blotting. Western blot analysis was performed as previously described in the text with the additional use of the following antibodies: 1: Lamp-2a (Abcam Ab18528),

Mouse organ dissection for biochemical analysis. Non-transgenic mice were anaesthetized and received a cardiac perfusion of cold PBS. The heart, lungs, liver, kidneys, spleen, brain, and spinal cord were then dissected out, rinsed in cold PBS, rapidly frozen on dry ice, and stored at -80°C until analysis.

Isolated Neural Cell Types. Astrocytes were cultured as described previously (Greco et al., 2010). Primary cortical and primary hippocampal neurons were gifts from Dr. Virginia Lee (University of Pennsylvania, Philadelphia, PA). Primary spinal cord neurons and purified motor neurons were gifts from Dr. Serge Przedborski (Columbia University, New York, NY). Precursor and mature oligodendrocytes were gifts from Dr. Judith Grinspan (Children's Hospital of Philadelphia, Philadelphia, PA).

Lysosomal Activity Assays with Lamp-2a antibody. Lysosomal activity assays with exogenous α-synuclein were performed as previously described in the text. For conditions in which the Lamp-2a antibody was added, the lysosomes were pre-incubated with 10ug of Lamp-2a antibody (Abcam18528) for 20 min at 37°C preceding addition of the α-syn substrate.

In gel digestion and MS/MS analysis. Duplicate samples of 50µg of lysosomes and lysosomal membranes purified as described above were mixed with 6X Laemmli sample buffer and separated on a 10% Bis-tris Novex Nupage gel (Invitrogen NP0301). One set of the lysosomes and lysosomal membranes was analyzed by western blot for Lamp-2a as described above. For the other set of lysosomes and lysosomal membranes, the gel was visualized by colloidal blue stain (Invitrogen LC6025). Stained gels were processed by in-gel trypsin digestion according to the following protocol. Slices were cut from the gel at apparent molecular weights corresponding to the Lamp-2a bands in the parallel western blot. Each slice was placed in single well of an Axygen plate and de-stained with 110 µl of de-stain buffer (50% methanol, 1% acetic acid, 49% water) overnight at room temperature on a rotator. Destained gel pieces were dehydrated in 100ul of acetonitrile (ACN) for 5min at room temperature. Acetonitrile was aspirated off and 5mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in 50mM ammonium bicarbonate buffer was added for 15 minutes at 37 ^{o}C in order to reduce cysteine thiols. Subsequently, cysteine thiols were alkylated with 50mM iodoacetamide in 25mM ammonium bicarbonate buffer for 30 minutes at 37 ^{o}C in the dark. Iodoacetimide treatment results in additional mass of 57 amu to the cysteine thiol and the carboxyamidomethylated cysteine is used as a static modification when the experimental data is searched against theoretical databases. In the next step the gel pieces were dehydrated with 100 µl acetonitrile and hydrated with 100 µl of 10mM AmBic for 5 minutes at room temperature. Next, 100 µl of acetonitrile were added for 5 minutes followed by 50 µl of 10mM AmBic containing 10ng/ul of trypsin. Trypsin digestion was performed for six hours at 37 ^{o}C. Tryptic peptides were extracted from the gel with 50ul of 0.3% formic acid for 2 hours at room temperature on a rotator. Supernatants (about 100 µl) were transferred to Axygen tubes and gel pieces were incubated with 50% ACN for additional two hours at room temperature. Liquid was recovered and combined with formic acid extracts. The volume was reduced to 5 µl with speedvacing and adjusted to the desired volume (about 15 µl) with 0.1% formic acid. Tryptic peptide digests were analyzed by hybrid LTQ-Orbitrap mass spectrometer (Thermo Electron, San Jose, CA) coupled to an Eksigent 2D LC system (Eksigent Technologies, Livermore, CA) and autosampler. Buffers A and B were 0.1 % formic acid/1 % methanol and 80% acetonitrile/0.1 % formic

acid/1 % methanol, respectively. Peptides were loaded isocratically onto a C_{18} trap column (75 um i.d. x 25 mm; New Objective Proteopep 2) at a flow rate of 1 μl per minute in 2% B. Peptides were then eluted onto a C_{18} analytical column (75 um i.d. x 150 mm; New Objective Proteopep 2). A linear gradient was then initiated at a flow rate of 300 nL per minute for 90 min from 3 - 40% B. The mass spectrometer was set to repetitively scan from 375 to 1600 m/z followed by data-dependent MS/MS scans on the five most abundant ions with dynamic exclusion enabled.

Generation and evaluation of SEQUEST peptide assignments. DTA files were generated from MS/MS spectra extracted from the RAW data file (intensity threshold of 2500; minimum ion count of 50) and processed by the ZSA and Correction algorithms of the SEQUEST Browser program. DTA files were submitted to Sorcerer-SEQUEST (ver. 4.0.3, rev 11; Sagen Research, San Jose, CA) using the following parameters: Database searching was performed against a Uniprot database (Release v3.57; 3/24/2009) containing *Mus musculus* sequences from Swiss-Prot plus common contaminants, which were then reversed and appended to the forward sequences. The database was indexed with the following parameters: mass range of 600 – 3500, tryptic cleavages with a maximum of 2 missed cleavage and static modifications of cysteine by carboxyamidomethylation (+57 amu). The DTA files were searched with a 50 ppm peptide mass tolerance for LTQ-Orbitrap, 1.0 amu fragment ion mass tolerance, and variable modification of methionine (+16 amu) for in-gel digestion, and maximum number of variable modifications of 3. Potential sequence-to-spectrum peptide assignments generated by Sorcerer-SEQUEST were loaded into Scaffold (version 2.2; Proteome Software, Portland, OR) to validate protein identifications. Protein identifications were accepted at a threshold of ≥ 99 % protein confidence with ≥ 2 unique peptides at ≥ 80 % confidence. These criteria resulted in an estimated protein false discovery rate (FDR) of ≤ 5 %, as calculated by the number of hits to the reverse protein sequences.

SUPPLEMENTARY TABLE 1.

Lamp-2 Peptides Detected in the 72kDa Fraction of Lysosomes and Lysosomal Membranes

Protein name	Protein accession numbers	Protein molecular weight (kDa)	Peptide sequence	SEQUEST XCorr score	SEQUEST DCn score	Observed Mass	Actual Mass	Delta AMU	Delta PPM
Lamp-2	P17047	45.681	$_{141}$VPLDVIFK$_{148}$	2.1628	0.2973	465.7865	929.5584	-0.00039	-0.4137
			$_{149}$CNSVLTYNLTPVVQK$_{163}$	3.8882	0.4604	868.4581	1,734.90	0.004173	2.404
			$_{286}$YLDFIFAVK$_{294}$	2.0993	0.2859	558.3127	1,114.61	0.004566	4.093
			$_{347}$AFQINTFNLK$_{356}$	2.6936	0.2084	598.3267	1,194.64	-0.001	-0.838

Bands corresponding to the 96kDa and 72kDa immunoreactive species of Lamp-2a were excised from a lane of purified lysosomes run on an SDS-PAGE gel and analyzed by mass spectrometry. Four different peptides corresponding to Lamp-2 were identified in the 72kDa band, but no Lamp-2 peptides were identified in the 96kDa band, reinforcing that the 72kDa band is the specific species corresponding to Lamp-2a.

SUPPLEMENTARY FIGURE LEGENDS

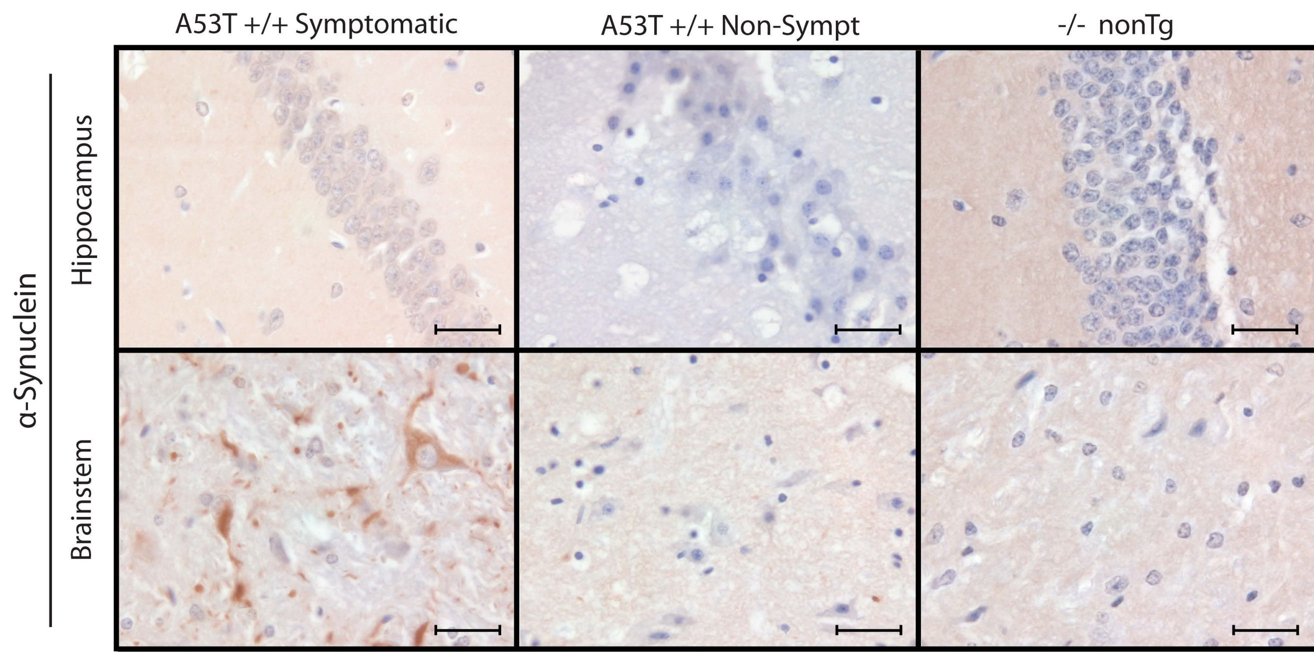
SUPPLEMENTARY FIGURE 1. Selective formation of α-syn inclusions in the brainstem of symptomatic mice. Immunohistochemical analysis with an antibody against human α-syn (Syn303) of sections from the hippocampus and the brainstem of symptomatic A53T α-syn transgenic mice, age matched non-symptomatic A53T α-syn transgenic mice, and age matched non-transgenic mice (-/- nonTg) confirmed that the brainstem of symptomatic mice does contain α-syn inclusions. Scale bar, 30μm.

SUPPLEMENTARY FIGURE 2. Characterization of Lamp-2a immunoreactivity. ***A,*** The brainstem, spinal cord, and remainder of the brain excluding these regions "Brain (–)BS" were dissected from non-transgenic littermates of the A53T α-syn mice or from Lamp-2 knockout mice. This tissue was analyzed by SDS-PAGE/western blot for Lamp-2a using the Abcam Ab18528 Lamp-2a antibody (top panel). The 96kDa band recognized by the antibody was present in the Lamp-2 knockout mice (asterisk), while the 72kDa band is specific to Lamp-2a (arrow). Fractionation of crude lysosomal extracts (Light M+L fraction) or purified lysosomes demonstrated that while the immunoreactivity of the Abcam

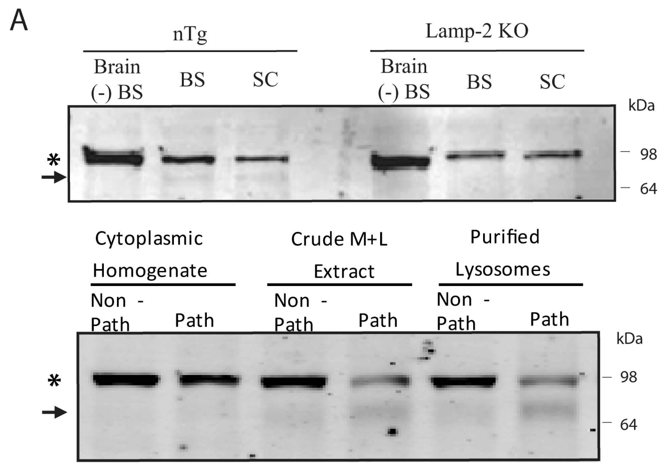
antibody for the 72kDa band was faint in whole homogenate, this band was enriched in lysosomes (bottom panel). ***B,*** Seven tissues of non-transgenic mice were analyzed by SDS-PAGE/western blot for Lamp-2a. The amount of protein loaded for each organ was adjusted to yield signals of relatively consistent intensity (Heart, 100µg; liver, 2µg; lung, 25µg; kidney, 5µg; spleen, 35µg; brain, 20µg; spinal cord, 50µg). ***C,*** Examination of different purified neural cell types by SDS-PAGE/western blot for Lamp-2a indicates that the neural 72kDa band of Lamp-2a is found in a variety of neural cell types. The amount of protein loaded for each cell type was adjusted to yield signals of relatively consistent intensity (Cortex, 30 µg; Hippocampus, 20 µg; Spinal Cord, 15 µg; Motor Neurons, 50 µg; Astrocytes, 1.5 µg; Precursor Oligodendrocytes, 1.5 µg; Mature Oligodendrocytes, 1.5 µg).

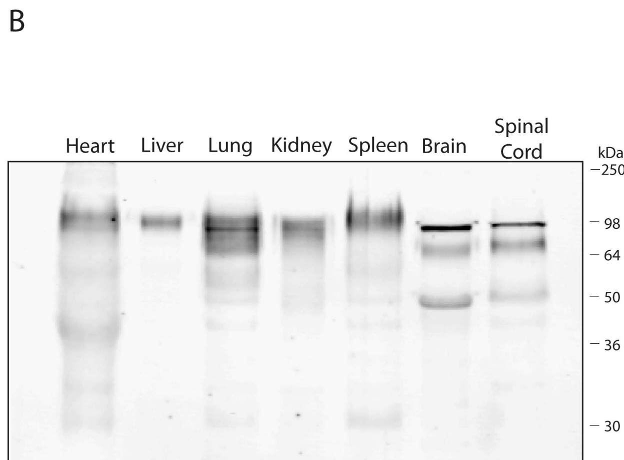
SUPPLEMENTARY FIGURE 3. Purified lysosomes were extracted from the pathogenic regions of the brainstem and spinal cord ("Path") or the remainder of the brain ("Non-Path") of non-transgenic mice. 25 µg of purified lysosomes were incubated with 0.2 µg of purified human α-syn with Hsc70 and an energy regenerating system at 37°C. To determine the role of Lamp-2a in this lysosomal uptake and degradation, one condition involved pre-incubation with an antibody against the C-terminal tail of Lamp-2a. A condition of lysosomes burst by hypotonic shock was included as a control for the activity of lysosomal proteases in the two samples. After the incubation, the samples were analyzed by SDS-PAGE/western blot with a human specific α-syn antibody and quantified by densitometry. All values were held relative to a condition in which the 0.2 µg of α-syn was incubated under the same conditions only without any lysosomes present. The Lamp-2a antibody significantly decreased the degradation of α-syn in the pathogenic regions compared to the non-pathogenic regions (**$p<0.05$ path versus non-path regions, t-test, n=2).

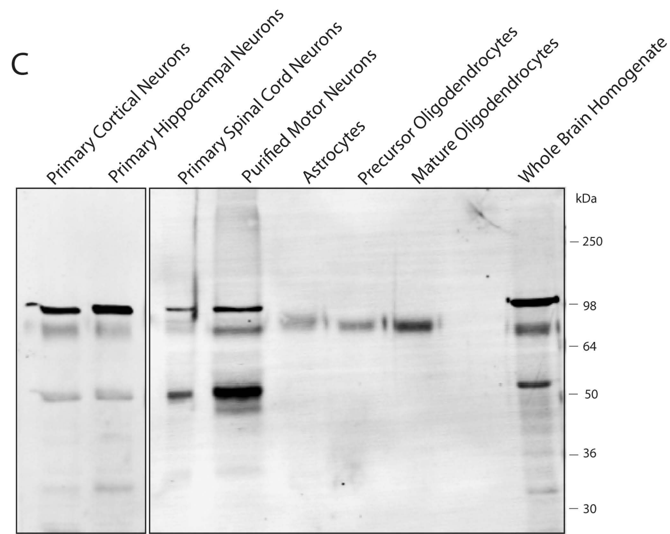
Supplementary Figure 1



Supplementary Figure 2







Supplementary Figure 3