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Hereditary spastic paraplegia (HSP) gene 11 (*Spg11*) attenuates lipid accumulation in myeloid cells and neuroinflammation in the midbrain without affecting α -synuclein pathology

Margaret Hayne¹, Joanna Lipka¹, Tawaun A. Lucas², Allison L. Soung¹, Max Adrian³, Soumitra Ghosh¹, Han Lin¹, Sarah Chu¹, Kimberly Stark¹, Hai Ngu³, Oded Foreman³, Joshua Kaminker² and Casper C. Hoogenraad^{1*}

Abstract

Hereditary spastic paraplegia type 11 (SPG11-HSP) is a neurodegenerative disorder caused by mutations in *SPG11*, which encodes the large scaffolding protein spatacsin, involved in lysosomal and autophagosomal trafficking. A portion of patients with *SPG11* mutations present with parkinsonism features. While spatacsin dysfunction is linked to neurodegeneration, the underlying cellular mechanisms, especially in the midbrain, remain largely unclear. Here, we demonstrate that loss of *Spg11* in mice results in neuroinflammation and lipid accumulation in myeloid cells. Bulk RNA sequencing revealed a strong upregulation of microglial genes in the midbrain of *Spg11* knockouts, supported by increased CD68 and CLEC7A expression and morphological changes consistent with microglial activation. *Spg11* depletion in two in vivo models of synucleinopathy revealed no enhancement of phosphorylated α -synuclein-positive inclusions or dopaminergic neuron loss; however, the mice did exhibit *Spg11*-dependent microglial reactivity. Further in vitro studies using primary bone-derived macrophages revealed increased phagocytic capacity and neutral lipid accumulation under basal and stress conditions. These findings support a model where SPG11 is a critical regulator of microglial activation and myeloid lipid metabolism, contributing to neurodegeneration through pathways distinct from α -synuclein-mediated pathology.

Highlights

- *Spg11* knockout activates microglia and induces lipid accumulation.
- Pathology observed in *Spg11* knockout persists in mouse models of synucleinopathy.
- RNA-seq shows robust upregulation of microglial genes in *Spg11* knockout midbrains.
- SPG11 deficiency alters microglial morphology and increases CD68 expression.
- Macrophages lacking SPG11 display enhanced phagocytosis and lipid storage.

Keywords SPG11, Hereditary spastic paraplegia, Synucleinopathy, Microglia, Neuroinflammation, Lipid metabolism

*Correspondence:
Casper C. Hoogenraad
hoogenraad.casper@gene.com

Full list of author information is available at the end of the article



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Introduction

Hereditary spastic paraplegia (HSP) represents a group of progressive neurodegenerative disorders characterized by progressive degeneration of the corticospinal tracts; clinically, HSP patients present with lower limb spasticity and progressive weakness [1–3]. HSP has been linked to mutations in more than 80 genes and can be described as either pure, meaning symptoms present only as motor deficits owing to the degeneration of corticospinal tracts, or complex, in which cases the motor manifestations is observed in conjunction with additional neurological symptoms such as cognitive impairment, ataxia, seizures, parkinsonism, or vision/hearing loss, depending on which genes are mutated [3–7]. Mutations in *SPG11* result in complex HSP and represent the most common form of autosomal recessive HSP worldwide [8]. Patients with HSP caused by *SPG11* mutations (SPG11-HSP) present with a variety of clinical signs and symptoms including gait abnormalities, intellectual disability, parkinsonism, and peripheral neuropathy [4, 8, 9]. At a structural level, thinning of the corpus callosum is characteristic of HSP patients with *SPG11* mutations, with the hyperintensity of the forceps minor termed the “ears of the lynx” sign [8, 10–12]. The disease is heterogeneous in presentation and progression, even among siblings [4, 8, 13–15]. Almost all mutations of *SPG11* which result in HSP appear to cause loss of gene function resulting from frameshift or nonsense mutations [16].

SPG11 encodes spatacsin, a large scaffolding protein, which plays a critical role in intracellular trafficking pathways, particularly those involving lysosomes and autophagosomes [17–21]. Spatacsin plays an essential role in lysosomal reformation and autophagic flux following starvation and stress conditions. These processes facilitate the clearance of cellular debris and damaged organelles, which is vital for cellular health and maintenance, particularly in neurons [22]. At the molecular level, spatacsin is part of the AP5 complex, along with AP5Z1 (mutations in which lead to SPG48-HSP) and spastizin (encoded by *SPG15*), which interacts with components of the endolysosomal system and the cytoskeleton to regulate membrane dynamics, vesicle trafficking, and lysosomal reformation [23]. Dysfunctional spatacsin, either through knockdown or mutations in patients, leads to an accumulation of undegraded lysosomal substrates and defective lipid metabolism in neurons, which contributes to neurodegeneration in HSP patients [19, 20, 24, 25].

As mentioned above, a subset of SPG11-HSP patients present with parkinsonism features, accompanied by a loss of neurons in the nigrostriatal region and an overall lower level of dopamine [15, 26–30]. Interestingly, the parkinsonism features in HSP patients respond to dopamine replacement therapies, suggesting a dysfunction

of nigrostriatal pathways in SPG11-HSP [15, 30, 31]. In one case study where an autopsy was performed on an SPG11-HSP patient, there was a loss of pigmented neurons and gliosis in the substantia nigra and locus coeruleus as well as lewy body-related α -synucleopathy despite the patient having no genetic mutations linked to α -synucleinopathy and only being 44 years old [32]. A second case study also observed neuronal loss and astrogliosis of the substantia nigra in an SPG11-HSP patient although no lewy bodies or α -synuclein inclusions were observed in this case [16]. Thus, the mechanism of parkinsonism in SPG11-HSP patients is not clearly understood and it is unknown if the impaired lysosomal function from loss of spatacsin function is acting through α -synuclein pathology in a similar mechanism to Parkinson's disease.

In addition to possible cell intrinsic mechanisms of neurodegeneration in the nigrostriatal pathway, there is also an increase in gliosis observed in patients, including both previously mentioned case studies, which indicates the possibility of inflammation-driven degeneration [16, 32]. While *SPG11* is ubiquitously expressed in the body, the vast majority of studies have focused on neurons, leaving the role of SPG11 in diverse cell types of the nervous system, including glia, unexplored. Glial cells play a crucial role in neurodegeneration by regulating neuroinflammation, maintaining neuronal health, and contributing to disease progression when their cellular functions become dysregulated. There have been a limited number of studies demonstrating that, in addition to dysfunction in neurons, *SPG11* mutations also lead to aberrations in microglial function, including increased reactivity and changes in lysosome numbers [33, 34].

In this study, we investigated the effects of *Spq11* loss on central nervous system pathology, with a focus on the midbrain due to its involvement in the parkinsonism features observed in SPG11-HSP patients. Using aged *Spq11* knockout mice, we uncovered a robust pro-inflammatory transcriptional signature driven primarily by microglial activation. Despite the association of parkinsonism with SPG11-HSP, we found that loss of SPG11 did not cause dopaminergic neuron degeneration in an aged model. In order to test the hypothesis that dopaminergic neuron loss is driven by α -synuclein aggregation, we crossed *Spq11*^{-/-} mice to those expressing human α -synuclein with an aggregation-prone A53T mutation. We also attempted to further challenge these animals by injecting pre-formed fibrils of α -synuclein into their substantia nigra. Neither of these α -synuclein models showed dopaminergic neuron loss or α -synuclein pathology. Instead, *Spq11* deficiency promoted neurodegenerative phenotypes independent of α -synuclein aggregation, including impaired motor function, increased neuronal injury markers, and reduced animal survival. Notably, it

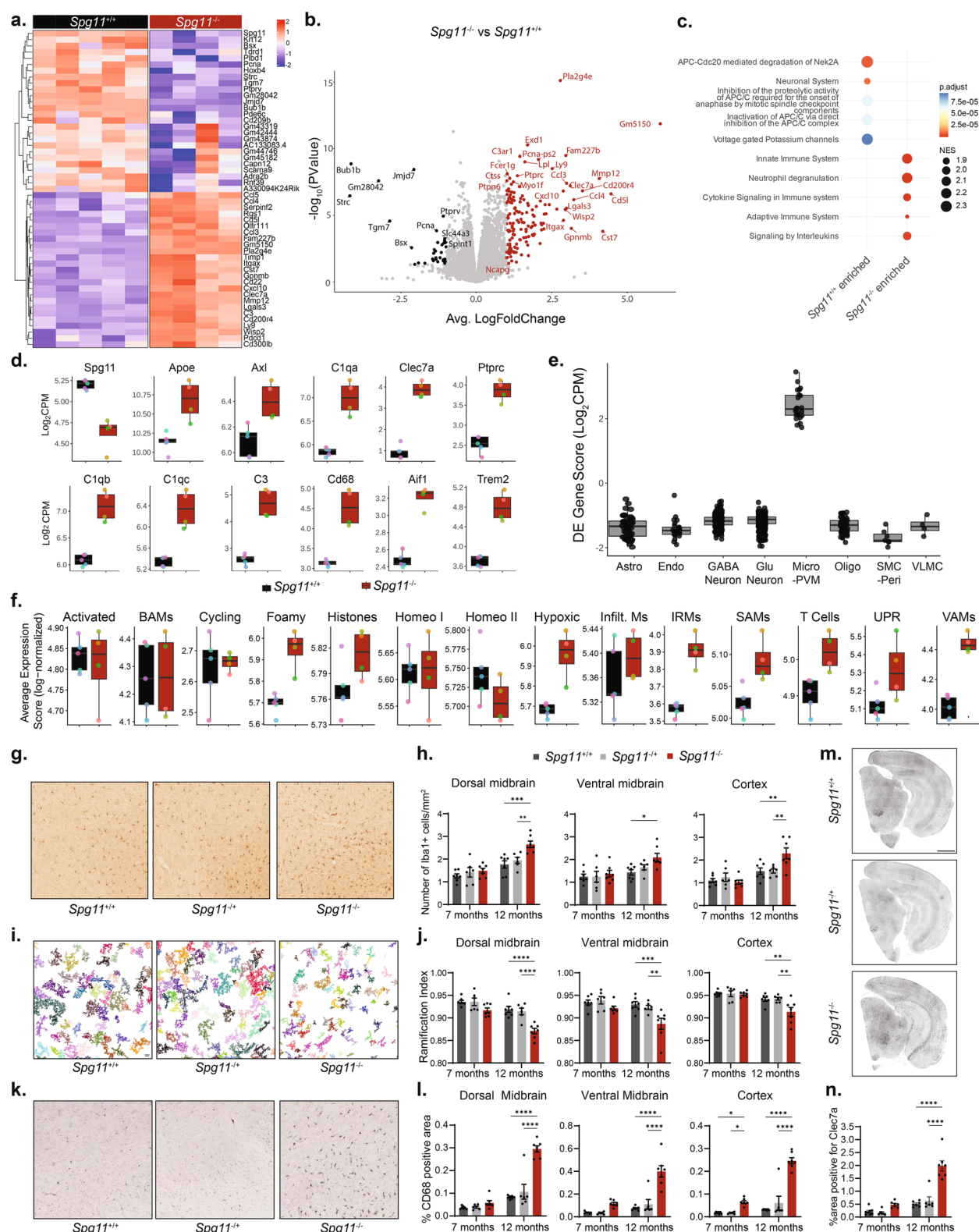


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Fig. 1 Genetic depletion of SPG11 in aged mice increases neuroinflammation. **a** Heatmap of differentially expressed genes between the midbrains of *Spg11*^{-/-} and *Spg11*^{+/+} mice aged 12 months. **b** Volcano plot comparing gene expression between *Spg11*^{-/-} and *Spg11*^{+/+} midbrains at 12 months. **c** GO term analysis of differentially expressed genes between *Spg11*^{-/-} and *Spg11*^{+/+} mice. **d** Log-fold change of selected microglia genes between *Spg11*^{-/-} and *Spg11*^{+/+} midbrains at 12 months. Each point represents a single animal. **e** Celltype score estimation calculated against publicly available Allen brain atlas single cell data. Pseudobulk counts were generated for each cell type for the DE genes between *Spg11*^{-/-} and *Spg11*^{+/+} samples, with a higher score indicating more DE genes predicted in that cell type. **f** Scores for expression of genes fitting into defined myeloid cell types in the midbrain of 12 month old *Spg11*^{+/+} and *Spg11*^{-/-} mice. Bams=border associated macrophages, Ims=interferon-responsive myeloid cells, Sams=synapse-associated myeloid cells, Vams=vasculature-associated myeloid cells. **g** Representative images of immunostaining against Iba1 in the ventral midbrain of *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 12 months old. **h** Quantification of the number of IBA1-positive cells per mm² across brain regions in *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 7 and 12 months old. Two way ANOVA with Tukey's multiple comparisons test, * $p \leq 0.05$, ** $p < 0.01$. Error bars are SEM. **i** Representative images of IBA1-positive cells segmented via watershed segmentation algorithm to calculate their ramification index in the ventral midbrain of *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 12 months old. Scale bar is 20 μ m. **j** Ramification scores of microglia across listed brain regions in *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 7 and 12 months old, where a lower ramification index implies a more amoeboid cell shape. Two way ANOVA with Tukey's multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars are SEM. **k** Representative images of CD68 staining in the ventral midbrain of *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 12 months old. **l** Quantification of the percent area positive for CD68 across brain regions in *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 7 and 12 months old. Two way ANOVA with Tukey's multiple comparisons test, **** $p < 0.0001$. Error bars are SEM. **m** Representative images of Clec7a staining in the brains of *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 12 months old. Scale bar is 1000 μ m. **n** Quantification of percent area of the brain positive for Clec7a in 7 month and 12 month old *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice. Two way ANOVA with Tukey's multiple comparisons test, **** $p < 0.0001$. Error bars are SEM

was also found that SPG11 regulates lipid metabolism in myeloid cells, and its depletion results in aberrant lipid accumulation, underscoring a potential mechanistic link between disrupted lipid handling and neuroinflammation. These data suggest that SPG11 preserves myeloid cell function under stress by regulating lipid biology. Impairment of this pathway leads to chronic inflammation, likely contributing to neurodegeneration independently of α -synuclein pathology.

Results

Spatacsin depletion promotes age-dependent microglial activation

A portion of SPG11-HSP patients exhibit parkinsonism features with some responding to dopamine replacement therapy, indicating a degeneration of the nigrostriatal pathway, but the mechanism of this degeneration remains unclear [27, 30, 31]. To investigate the role of spatacsin in this brain region, we performed bulk RNA sequencing on midbrain tissue from 12 month old *Spg11*^{+/+} and *Spg11*^{-/-} mice. Differential gene expression analysis revealed an upregulation of proinflammatory genes in *Spg11*^{-/-} midbrains (Fig. 1a–d). This included complement pathway genes (*C1qa*, *C1qb*, *C1qc*, *C3*), phagocytic markers (*Cd68*, *Clec7a*, *Axl*), and DAM-marker genes (*Apoe*, *Trem2*, *Ptprc*, *Aif1*). Broader analysis also indicated enhanced signaling through immune-related GO terms in *Spg11*^{-/-} animals (Fig. 1c). These GO terms included defense response to other organisms and symbionts, the innate immune response, and positive regulation of immune system responses. To identify the cell types most affected by these transcriptional changes, we examined the expression of the differentially expressed genes in a single-cell RNA-seq reference dataset, which highlighted microglia as the most impacted population (Fig. 1e). We found minimal differences in other cell types using this method, including astrocytes, neurons, and oligo lineage

cells. To better characterize changes in myeloid cell state in *Spg11*^{-/-} brains, we used the geneset defining microglia states to map the relative expression of each state, finding an increase in foamy, hypoxic, and vasculature-associated myeloid cells in *Spg11*^{-/-} midbrains compared to *Spg11*^{+/+} (Fig. 1f). These findings suggest that *Spg11* loss of function in aged mice leads to specific activation of microglia compared to changes in other cell types in the midbrain.

To validate the microglial activation signature observed in our RNA sequencing data, we performed histological analysis in *Spg11*^{-/-} mice. Given that neurodegeneration in HSP is progressive, we also assessed whether microglial phenotypes in *Spg11*^{-/-} mice evolved with age. We immunostained brain sections from *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 7 months and 12 months old using IBA1, a marker of microglia (Fig. 1g). We observed a significant increase in the number of IBA1-positive cells only in the older *Spg11*^{-/-} mice (12 months), with no differences detected in either the younger cohort or *Spg11*^{-/-} animals (Fig. 1h). To assess microglial morphology, we applied a watershed segmentation to isolate individual cells and quantified their ramification index, where decreased branching is indicative of a more activated state (Fig. 1i) [35]. This analysis revealed a significant reduction in microglial ramification in the dorsal midbrain, ventral midbrain, and cortex of *Spg11*^{-/-} mice at 12 months, consistent with a shift toward a reactive phenotype (Fig. 1j). To further characterize the microglial response to spatacsin loss, we performed immunostaining for CD68, a lysosomal marker of phagocytic and activated microglia (Fig. 1k). CD68 is particularly relevant given previous reports linking SPG11 to lysosomal function, including in microglia [34]. We observed a significant increase in CD68-positive area in *Spg11*^{-/-} mice across all analyzed brain regions, the dorsal midbrain, ventral midbrain, and cortex, relative to both wildtype

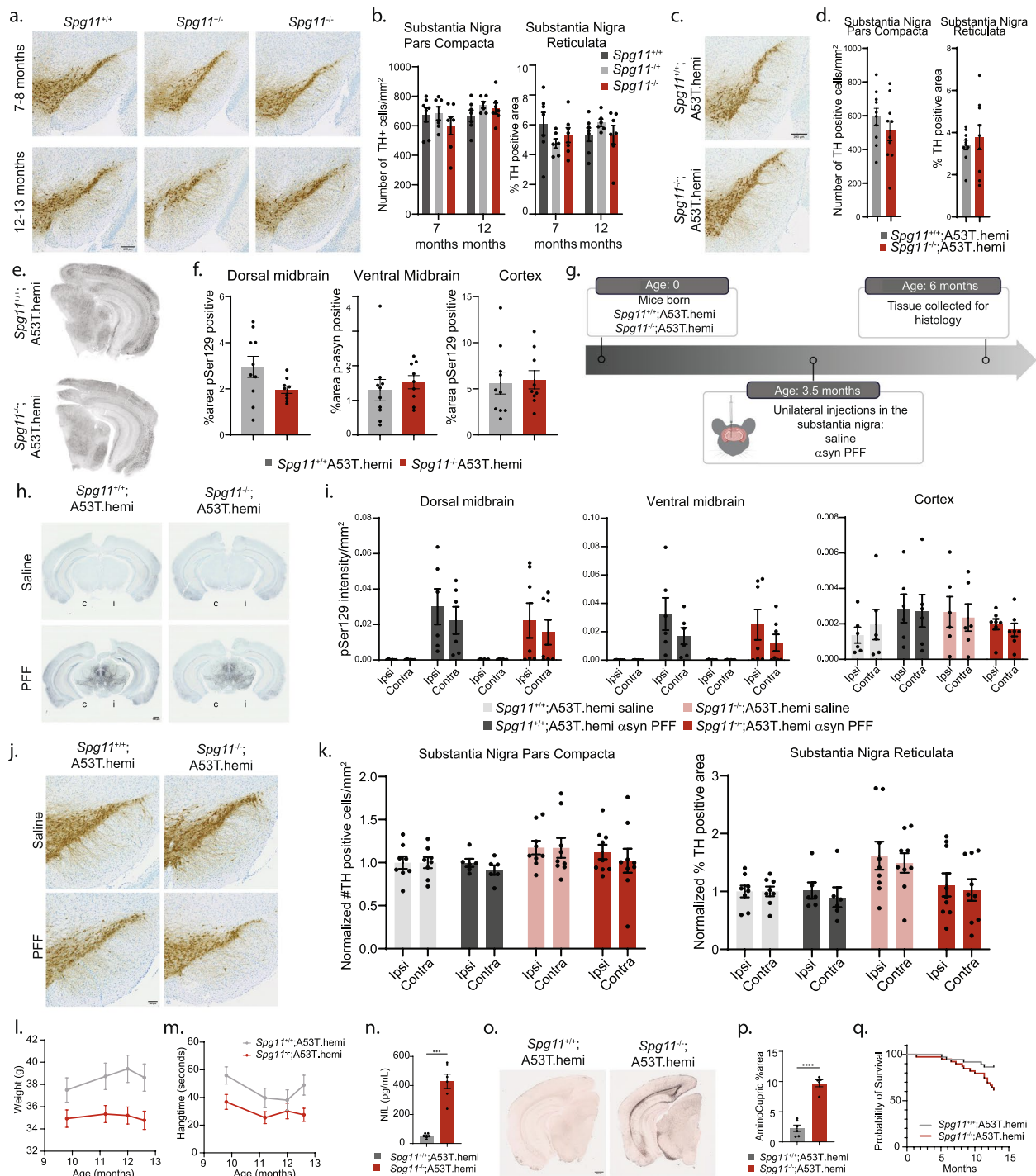


Fig. 2 (See legend on next page.)

and heterozygous controls. Notably, this increase was more robust in aged animals (12 months), while in younger mice (7 months), significant elevation of CD68 staining was restricted to the cortex (Fig. 1l). We also examined the percent area of the whole brain positive for Clec7a, and found a significant increase in *Spg11*^{-/-} animals at 12 months old, but not at age 7 months (Fig. 1m,

n). Together, these data suggest that loss of SPG11 promotes age-dependent changes to myeloid cell populations. *Spg11*^{-/-} mice did not differ from *Spg11*^{+/+} in any measures, indicating that partial spatacsin function is sufficient to maintain normal microglial homeostasis.

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Fig. 2 Loss of SPG11 does not affect α -synuclein accumulation or dopaminergic neuron vulnerability in two models of pathology, but does increase other neurodegeneration. **a** Representative images immunostaining against Tyrosine hydroxylase (TH) of the substantia nigra region of *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 7 and 12 months old. Scale bar is 200 μ m. **b** Quantification of the number of TH-positive neurons in the substantia nigra by both cell body count (number of cells/area) in the pars compacta and by axon density (optical density/area) in the reticular region. Analyzed by two way ANOVA, not significant. Error bars are SEM. **c** Representative images immunostaining against Tyrosine hydroxylase (TH) of the substantia nigra region of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. Scale bar is 200 μ m. **d** Quantification of the number of TH-positive neurons in the substantia nigra by both cell body count (number of cells/area) in the pars compacta and by axon density (optical density/area) in the reticular region. Analyzed by two way ANOVA, not significant. Error bars are SEM. **e** Representative images of brains stained against α -synuclein phosphorylated at serine 129 (pSer 129) from *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. **f** Quantification of the percent area positive for pSer 129 α -synuclein across dorsal midbrain, ventral midbrain, and cortex of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. Analyzed by two way ANOVA, not significant. Error bars are SEM. **g** Schematic of timeline for *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice injected unilaterally in the substantia nigra with either saline or α -synuclein pre-formed fibrils (PFFs) at 3.5 months old. Mice were allowed to age after injection and tissue was collected for analysis when they were 6 months old, 2.5 months after injection. **h** Representative images of brains stained against α -synuclein phosphorylated at serine 129 (pSer 129) from *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice unilaterally injected with saline or α -synuclein PFFs. c = contralateral, i = ipsilateral. Scale bar is 500 μ m. **i** Quantification of the percent area positive for pSer 129 α -synuclein across dorsal midbrain, ventral midbrain, and cortex of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice unilaterally injected with saline or α -synuclein PFFs. Analyzed by two way ANOVA, not significant. Error bars are SEM. **j** Representative images immunostaining against Tyrosine hydroxylase (TH) of the substantia nigra region of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice injected with saline or α -synuclein PFFs. Images show substantia nigra in the side ipsilateral to injection. Scale bar is 100 μ m. **k** Quantification of the number of TH-positive neurons in the substantia nigra by both cell body count (number of cells/area) in the pars compacta and by axon density (optical density/area) in the reticular region. Analyzed by two way ANOVA, not significant. Error bars are SEM. **l** Weights of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice as they age. Error bars are SEM. **m** Latency to fall off wire grate during hangtime test for *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice across time. Error bars are SEM. **n** Concentration of NfL in serum of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. Analyzed by T-test. Error bars are SEM. **o** Representative images of brains from *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old stained with amino cupric to show degenerating neurites. Scale bar is 500 μ m. **p** Quantification of percent area of total brain positive for amino cupric staining. Analyzed by T-test, error bars are SEM. **q** Survival graph showing percentage of cohort surviving over the 13 months of the study for *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice

***Spg11* depletion does not modulate α -synuclein aggregation or dopaminergic neuron integrity**

To investigate whether the nigrostriatal pathway is impaired in this model of SPG11-HSP, we first assessed the integrity of the dopaminergic system in *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} mice at 7 months and 12 months old by immunostaining for tyrosine hydroxylase (TH), a marker of dopaminergic neurons (Fig. 2a). We found no significant differences in the number of TH-positive cell bodies in the substantia nigra pars compacta or in axonal density in the substantia nigra reticulata between genotypes at either age (Fig. 2b).

Given the absence of spontaneous dopaminergic neurodegeneration in the substantia nigra of *Spg11*^{-/-} mice, we next asked whether spatacsin loss would exacerbate pathology in a chronic model of α -synuclein stress, which we had previously used [36]. We crossed mice expressing a single copy of human α -synuclein carrying the A53T mutation (A53T.hemi) with *Spg11*^{+/+} and *Spg11*^{-/-} mice and aged them to 13 months old. Brains were then examined by immunostaining against TH to assess dopaminergic neuron loss in the substantia nigra and for phosphorylated α -synuclein at serine 129 (pSer129), a marker associated with aggregation-prone, pathological α -synuclein (Figs. 2c, e). We observed no significant differences between *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice in either TH-positive neuron counts or pSer129 α -synuclein levels (Figs. 2d, f). We also used bulk RNA sequencing of the midbrains of these mice as an alternative method to test for dopaminergic neuron loss or changes to the integrity of neurons in the

nigrostriatal pathway. We found no *Spg11*-dependent differences in gene expression related to dopamine biology (Supplementary Fig. 1a, b) or neuronal integrity (Supplementary Fig. 1c, d).

To further test whether spatacsin loss increases the brain's susceptibility to α -synuclein pathology following a more acute insult, we performed unilateral injection of pre-formed fibrils (PFFs) of α -synuclein into the substantia nigra. Injections were performed on *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 3 months of age, then mice were allowed to age to 6 months old (Fig. 2g). Similar models of PFF injection and spreading have been previously used and demonstrate a spreading pSer129 signal and dopaminergic cell loss [37]. The pathological index of *SNCA*^{A53T} transgenic mice is thought to be altered by the level of α -synuclein, which can be increased by either the copy number of the A53T overexpression construct or by increasing the age of the animal [38, 39]. We opted to use a mild model to better observe any potentiation resulting from *Spg11* loss of function, as PFF-injection models in older or homozygous *SNCA*^{A53T} animals show severe phenotypes including paralysis and death [40]. We predicted that *Spg11*^{-/-} would worsen any phenotypes from the baseline and that we would not be able to observe this in a model that was already toxic enough to cause lethality. Brains were analyzed for α -synuclein pathology using pSer129 immunostaining (Fig. 2h) and for dopaminergic neuron integrity in the substantia nigra using TH immunostaining in both hemispheres (Fig. 2j). As expected, PFF-injected animals showed elevated pSer129 staining in the



no significant difference in TH-positive neuron counts between *Spg11*^{+/-};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice following PFF injection. There was no differences in dopaminergic neurons in the substantia nigra between

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Fig. 3 Loss of SPG11 increases neuroinflammation in the presence of mutant α -synuclein. **a** Heatmap of differentially expressed genes between the midbrains of *Spg11*^{-/-};A53T.hemi and *Spg11*^{+/+};A53T.hemi mice at 13 months old. **b** Volcano plot comparing gene expression between *Spg11*^{-/-};A53T.hemi and *Spg11*^{+/+};A53T.hemi midbrains of 13 month old mice. **c** GO term analysis of differentially expressed genes between *Spg11*^{-/-};A53T.hemi vs. *Spg11*^{+/+};A53T.hemi mice at 13 months old. **d** Log-fold change of selected microglia genes between and *Spg11*^{-/-};A53T.hemi and *Spg11*^{+/+};A53T.hemi midbrains at 13 months old. Each point represents a single animal. **e** Scores for expression of genes fitting into defined myeloid cell types in the mid-brain of 12 month old *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice. Bams=border associated macrophages, Ims=interferon-responsive myeloid cells, Sams=synapse-associated myeloid cells, Vams=vasculature-associated myeloid cells. **f** 4 way plot comparing differentially expressed genes in *Spg11*^{-/-} vs. *Spg11*^{+/+} and *Spg11*^{-/-};A53T.hemi vs. *Spg11*^{+/+};A53T.hemi datasets. **g** Representative images of IBA1-positive cells segmented via watershed segmentation algorithm to calculate their ramification index in the ventral midbrain of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. Scale bar is 50 μ m. **h** Ramification scores of microglia across listed brain regions in *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old, where a lower ramification index implies a more ameboid cell shape. Two way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$. Error bars are SEM. **i** Representative images of brains of 13 month old *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice immunostained against CLEC7A. Scale bar is 500 μ m. **j** Quantification of the percentage of total cells that are CLEC7A positive in total brain for *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi 13 month old mice. Analyzed by T-test, **** $p < 0.0001$. Error bars are SEM. **k** Hemibrains of 13 month old *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice were analyzed by flow cytometry. This shows quantifications of the percentage of monocytes (CD45⁺ and CD11b⁺) cells which are also positive for P2RY12, CD11c, CD80, MHCII, and CLEC7A for both genotypes. Analyzed by T-test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars are SEM. **l** Primary bone marrow derived macrophages from *Spg11*^{+/+} and *Spg11*^{-/-} mice either negative or hemizygous for *SNCA*^{A53T} were treated with pHrodo-labeled zymosan particles for 16 h to measure phagocytosis. Shown here is quantification of the number of red-positive cells normalized to confluence of total cells in that well. All groups were again normalized to the average red count/confluence of *Spg11*^{+/+};A53T.neg BMDMs after 16 h (total phagocytosis of wildtype cells). Analyzed by two way ANOVA, * $p < 0.05$. Error bars are SEM

saline- and PFF-injected groups, and we hypothesize this is due to the limited accumulation of human α -synuclein in hemizygous A53T mice at this early age (Fig. 2k). In summary, our results indicate that *Spg11* loss of function does not sensitize dopaminergic neurons to degeneration or enhance α -synuclein pathology in either a chronic genetic model aged to 13 months or a more acute PFF-induced model 3 months after injection.

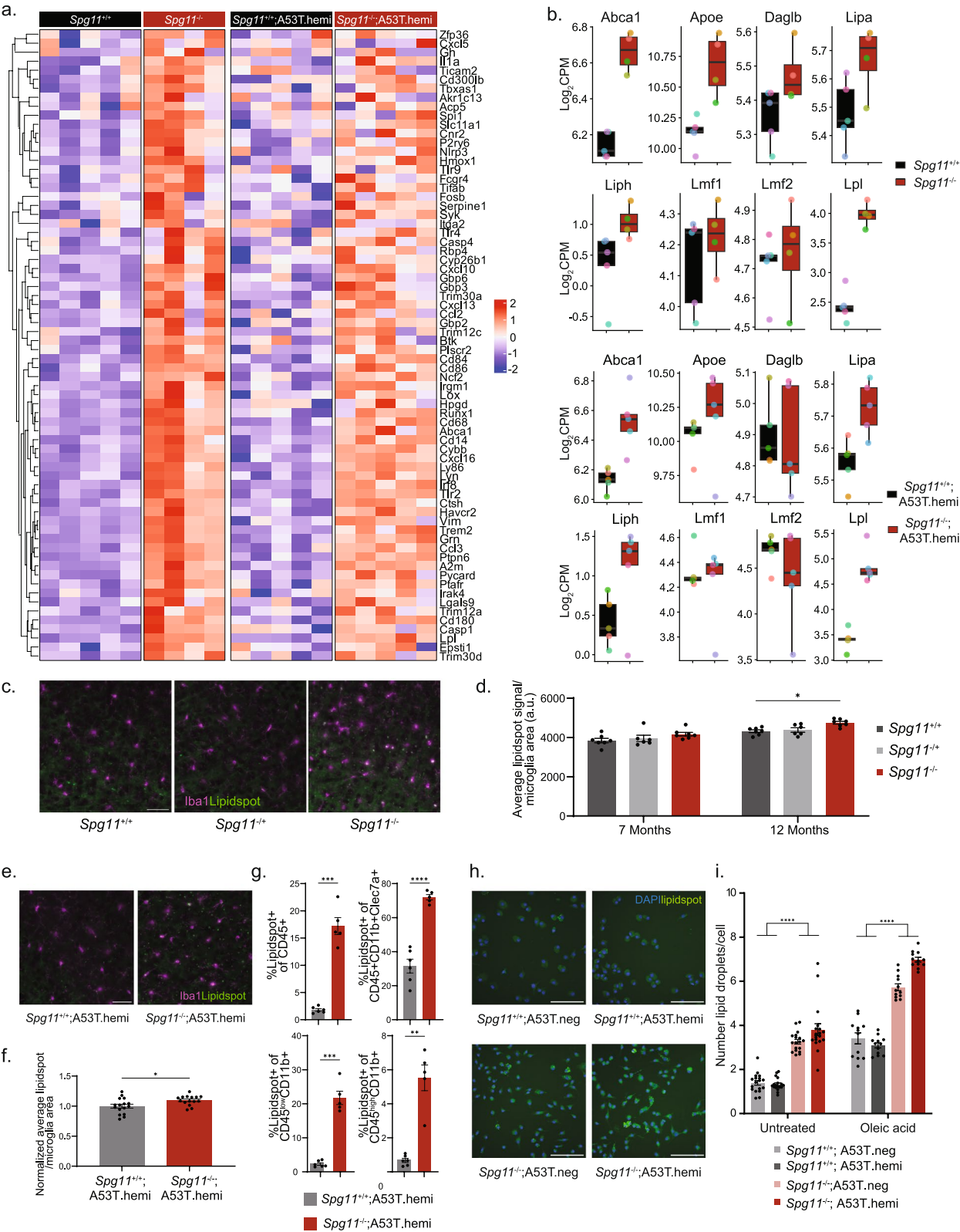
Spatacsin depletion promotes neurodegeneration and shortened lifespan

To investigate whether spatacsin loss induces neurodegeneration beyond that triggered by α -synuclein-related pathology, we assessed motor function in our chronic model of α -synuclein challenge using *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice. Throughout the study, *Spg11*^{-/-};A53T.hemi mice consistently exhibited lower body weight compared to their *Spg11*^{+/+};A53T.hemi counterparts, suggesting possible impairment (Fig. 2l). To assess motor strength and coordination, we conducted a hang time test at multiple time points over the course of aging. When considering all trials, *Spg11*^{-/-};A53T.hemi mice performed significantly worse than *Spg11*^{+/+};A53T.hemi mice, although no significant differences were observed at individual time points (Fig. 2m). To test whether this impairment was linked to neurodegeneration, we measured plasma levels of neurofilament light chain (NfL), a biomarker of neuronal injury, and assessed brain pathology via amino cupric silver staining for dystrophic neurites across the cerebrum. *Spg11*^{-/-};A53T.hemi mice showed elevated plasma NfL levels at 13 months compared to *Spg11*^{+/+};A53T.hemi mice (Fig. 2n), along with increased amino cupric reactivity (Fig. 2o, p), suggesting ongoing neurodegeneration. Furthermore, we observed reduced survival in the *Spg11*^{-/-};A53T.hemi group, with only 61.5% of mice surviving to 13 months

compared to 86.5% in the *Spg11*^{+/+};A53T.hemi group (Fig. 2q). These behavioral defects are of similar severity to the published effects of *Spg11* knockout in otherwise wild type mice [20], but we cannot assess whether α -synuclein pathology worsened these phenotypes without controls lacking A53T.hemi expression. Collectively, these results indicate that spatacsin plays a critical role in maintaining neuronal integrity, and its loss promotes neurodegeneration with minimal impact on the dopaminergic system or α -synuclein aggregation.

Spg11 loss of function is sufficient to increase neuroinflammation in the context of pathological α -synuclein expression

We next asked whether the presence of pathogenic α -synuclein would alter the pro-inflammatory gene expression profile we previously observed in *Spg11*^{-/-} mice. We hypothesized that α -synuclein pathology might act as a dominant immune trigger, either masking or exacerbating the effects of *Spg11* loss of function. To test this, we performed bulk RNA sequencing on the midbrains of 13-month-old *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice. The analysis revealed that most upregulated differentially expressed genes were pro-inflammatory (Fig. 3a-d). We also used the genesets which define myeloid cell states in the brain to show that there is an increase in the expression of genes defining foamy and vasculature-associated myeloid cells in *Spg11*^{-/-};A53T.hemi midbrains, as well as a decrease in the homeostatic II geneset (Fig. 3e). Importantly, we also compared the differentially expressed genes between *Spg11*^{-/-} vs. *Spg11*^{+/+} and *Spg11*^{-/-};A53T.hemi vs. *Spg11*^{+/+};A53T.hemi mice (Fig. 3f). We found 24 genes that were differentially expressed in both groups, and a general increase in differentially expressed genes found in the *Spg11*^{-/-} vs. *Spg11*^{+/+} dataset (94 genes that were



(See figure on previous page.)

Fig. 4 Loss of SPG11 increases lipid accumulation in myeloid cells, both *in vivo* and *in vitro*. **a** Heat map of the top 50 most differentially expressed lipid metabolism-related genes in the midbrain of *Spg11*^{+/+} and *Spg11*^{-/-} mice at 12–13 months old and in the midbrain of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. **b** Expression of a set of lipid genes in the midbrain of *Spg11*^{+/+} and *Spg11*^{-/-} mice at 12 months old and in the midbrain of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice at 13 months old. Each point represents one animal. **c** Representative image of brains stained against Iba1 and incubated with Lipidspot dye in 12 month old *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} animals. Scale bar is 50 μ m. **d** Quantification of the average intensity of Lipidspot dye in Iba1-positive cells in whole brains of *Spg11*^{+/+}, *Spg11*^{-/-}, and *Spg11*^{-/-} animals. Analyzed by two way ANOVA with Tukey's multiple comparisons, * $p < 0.05$. Error bars are SEM. **e** Representative images of brains stained against Iba1 and incubated with Lipidspot dye in *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice. Scale bar is 50 μ m. **f** Quantification of relative fold change from *Spg11*^{+/+};A53T baseline of average Lipidspot intensity in Iba1-positive cells in whole brains for *Spg11*^{+/+};A53T and *Spg11*^{-/-};A53T animals. Analyzed by Welch's T-test, * $p < 0.05$. Error bars are SEM. **g** Hemibrains of *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi animals were analyzed by flow cytometry for whether cells were lipidspot positive in various myeloid cell subpopulations. Analyzed by T-test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars are SEM. **h** Representative images of lipidspot staining in primary bone marrow-derived macrophages from *Spg11*^{+/+} and *Spg11*^{-/-} mice negative for or expressing a single copy of A53T α -synuclein. Cells shown here are untreated. Scale bars are 100 μ m. **i** Quantification of the number of lipid droplets per cell in primary bone marrow-derived macrophages from *Spg11*^{+/+} and *Spg11*^{-/-} mice negative for or expressing a single copy of A53T α -synuclein. One condition was untreated and the other was treated for 24 h with oleic acid, which increases lipid deposition as expected. Analyzed by two way ANOVA, **** $p < 0.0001$. Error bars are SEM

differentially expressed only in this dataset). This pattern supports a model where baseline inflammation is increased in animals expressing *SNCA*^{A53T}, but that loss of spatacsin is still sufficient to drive increased inflammation even in this immune-reactive environment. To further investigate the immune activation associated with spatacsin loss, we immunostained brains from the synucleinopathy models using microglial markers. IBA1 staining revealed reduced microglial ramification in *Spg11*^{-/-};A53T.hemi mice compared to *Spg11*^{+/+};A53T.hemi, indicating a more activated state (Fig. 3g, h). Immunostaining through the whole cerebrum against Clec7a, a marker of activated microglia, revealed a significant increase in *Spg11*^{-/-};A53T.hemi compared to *Spg11*^{+/+};A53T.hemi mice (Fig. 3i, j), including having a genotype effect in the younger injection model animals (Supplementary Fig. 2e, f). Brains from *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice injected with saline or PFFs were immunostained against CD68. CD68 staining showed increased microglial reactivity in the ventral midbrain of *Spg11*^{-/-};A53T.hemi mice injected with saline, with similar trends in other regions, particularly contralateral to the injection site. No genotype differences were observed in PFF-injected brains, suggesting that PFF-induced inflammation may be stronger than *Spg11* effects (Supplementary Fig. 1). Overall, these findings indicate that spatacsin loss is sufficient to enhance microglial activation within the context of α -synuclein pathology.

To further characterize how spatacsin loss affects myeloid cells, we used flow cytometry to analyze immune cell populations in hemi brains of 13-month-old *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice. The myeloid cell population was identified by cells that were positive for both CD45 and CD11b. The percentage of these cells, which were P2RY12+, an indication of resting homeostatic microglia, was significantly lower in *Spg11*^{-/-};A53T.hemi mice than *Spg11*^{+/+};A53T.hemi. Additionally, the myeloid cell population, which was positive for CD11c, CD80, MHCII, and CLEC7A, was

higher in the brains of mice lacking SPG11 (Fig. 3k). These findings indicate a shift from a homeostatic to an activated immune state in the CNS following *Spg11* deletion. To determine whether this activation reflects a cell-intrinsic role of SPG11 in myeloid cells or is secondary to neurodegeneration, we cultured primary bone marrow-derived macrophages (BMDMs), a readily accessible myeloid cell type from adult animals, from *Spg11*^{+/+} and *Spg11*^{-/-} mice, with or without A53T α -synuclein expression. *Spg11*^{-/-} BMDMs showed increased phagocytosis of pHrodo-zymosan particles (Fig. 3l). There was no effect from α -synuclein expression, which was not surprising as α -synuclein is not strongly expressed in BMDMs, but does indicate that exposure to pathogenic material in the mice prior to collection did not put the macrophages in any primed state. These results suggest that *Spg11* loss of function enhances myeloid cell reactivity in a cell-autonomous manner consistent with our *in vivo* observations.

Spatacsin regulates lipid metabolism in myeloid cells

Spatacsin has been previously implicated in lipid processing in neurons, and emerging evidence suggests that lipid metabolism in myeloid cells plays a key role in regulating immune responses [20, 41, 42]. To explore whether *Spg11* also influences lipid-related pathways in the brain's immune cells, we analyzed transcriptomic changes in *Spg11*^{-/-} mouse midbrains, including those hemizygous for A53T α -synuclein. We found a consistent upregulation of lipid-related genes across both cohorts (Fig. 4a, b). Upregulated genes included the cholesterol efflux transporter *Abca1*, the lipoprotein lipase *Lpl*, which represents a rate-limiting step triglyceride-rich lipoproteins, and the lipoprotein binding partner *Apoe*, which assists in lipid internalization. In addition to these individual genes which were upregulated in all animals, there was also a pattern of lipid processing genes which were generally upregulated with loss of spatacsin, but seemed to be less consistent across individual animals indicating a general trend in changes to lipid processing. Importantly, the upregulation of these genes followed the same

pattern in *Spg11*^{-/-} vs. *Spg11*^{+/+} and *Spg11*^{-/-};A53T.hemi vs. *Spg11*^{+/+};A53T.hemi animals, indicating that this effect comes from loss of spatacsin function and persists in the context of α -synuclein pathology. To test whether these transcriptional changes corresponded to altered lipid accumulation in brain immune cells, we co-stained brains of *Spg11*^{+/+}, *Spg11*^{-/-}, *Spg11*^{+/+};A53T.hemi, and *Spg11*^{-/-};A53T.hemi mice with Iba1, to denote microglia, and Lipidspot dye, to measure neutral lipid accumulation. We found a significant increase in microglial lipid accumulation in both 12 month old *Spg11*^{-/-} and *Spg11*^{-/-};A53T.hemi mice (Fig. 4c-f). Interestingly, we did not observe any differences in 7 month old *Spg11*^{-/-} mice (Fig. 4, c, d) or between any of the groups in our PFF-injection model which are 6 months old (Supplementary Fig. 3), which suggests that *Spg11*-dependent changes in microglial lipid processing develop with age. In order to more closely assess lipid levels in brain immune cells, we performed flow cytometry on hemi-brain single-cell suspensions from *Spg11*^{+/+};A53T.hemi and *Spg11*^{-/-};A53T.hemi mice. We observed a significant increase in Lipidspot-positive cells, indicative of neutral lipid accumulation, across several immune cell populations including total immune cells (CD45⁺), resting myeloid cells (CD11b⁺CD45^{low}), and activated myeloid cells (CD11b⁺,CD45^{high} and CD45⁺,Clec7a⁺) in *Spg11*^{-/-};A53T.hemi animals (Fig. 4c). Together, these results demonstrate that *Spg11* expression is required for proper lipid handling in brain-resident immune cells. To test whether the observed lipid accumulation was cell-intrinsic, we examined BMDMs from *Spg11*^{+/+} and *Spg11*^{-/-} mice, both with and without A53T α -synuclein overexpression. *Spg11*^{-/-} BMDMs consistently exhibited more lipid droplets per cell compared to *Spg11*^{+/+}, regardless of α -synuclein status. Upon oleic acid treatment, lipid accumulation increased in all groups as expected, with *Spg11*^{-/-} cells showing higher levels than *Spg11*^{+/+} (Fig. 4d, e). These data indicate that spatacsin is essential for lipid metabolism in myeloid cells and that its loss leads to aberrant lipid accumulation via a cell-intrinsic mechanism.

Discussion

In this study, we used *Spg11*^{-/-} mice as a model of hereditary spastic paraplegia to examine cellular changes in the midbrain. We found, using transcriptomic, histological, and cellular analyses of the midbrain, that *Spg11* loss of function leads to significant age-dependent microglial activation in the midbrain characterized by increased expression of pro-inflammatory genes, enhanced CD68, Iba1, and Clec7a reactivity, and reduced microglial ramification. Flow cytometry revealed a shift from homeostatic to activated myeloid populations, and primary *Spg11*^{-/-} macrophages showed increased phagocytic

activity, suggesting that spatacsin plays a cell-intrinsic role in regulating myeloid cell activation. Furthermore, spatacsin deficiency led to the accumulation of neutral lipids in both brain-resident and cultured immune cells, supporting a model where spatacsin is an essential regulator of lipid metabolism in myeloid cells [20]. These data also fit with a proposed role for spatacsin in modulating endolysosomal trafficking across cell types [22]. Myeloid cells, having a large phagocytic capability, especially rely on this pathway to break down material and efficiently exchange membrane lipids across compartments, where loss of *Spg11* function can lead to dysregulation of both processes. Our findings highlight the function of spatacsin in maintaining microglial homeostasis and suggest that dysregulated immune activity may underlie a portion of the neurodegeneration in HSP patients.

α -synuclein biology and dopaminergic neuron resilience in the substantia nigra are not affected by *Spg11* deletion

A subset of HSP patients exhibit parkinsonism features and SPG11 deletion has been associated with lysosomal dysfunction and lipid accumulation, pathways commonly disrupted in Parkinson's disease (PD) which also increases nigrostriatal neurodegeneration. Therefore, we had hypothesized that *Spg11* loss of function may cause vulnerability in dopaminergic neurons or aberrant α -synuclein processing via lipid or lysosomal dysfunction. Other risk factors for PD have been shown to play a role in lysosomal processing and lipid metabolism such as mutations in GBA1, which encodes a lysosomal enzyme that breaks down the sphingolipid glycosylceramide [43]. Increases in glucosylceramide and mutations in GBA1 in animal models have been demonstrated to increase α -synuclein aggregation [44, 45]. In studies modeling HSP through knockout of *Spg11*, an increase in gangliosides in lysosomes has been observed, which is proposed to contribute to cell death [19, 20, 25]. These studies went on to find that treatment with a glucosylceramide synthase inhibitor was sufficient to protect neurons from cell death. In addition, spatacsin localizes to the lysosome, and *Spg11*^{-/-} mice show an early accumulation of autofluorescent material in LAMP1-positive vesicles as young as 6 weeks old, indicating an inefficiency in the cellular compartment [20]. Therefore, we hypothesized that the toxic buildup of lipids and inefficient lysosomal processing in neurons lacking spatacsin would also increase α -synuclein aggregation, a process which would link HSP and PD pathogenesis. To test this hypothesis, we used two models of synucleinopathy, a chronic model with transgenic expression of A53T human α -synuclein in aged mice and an acute model using direct pre-formed fibril injection in the substantia nigra. Neither model showed an exacerbation of accumulation of phosphorylated α -synuclein or dopaminergic neuron loss with

deletion of *Spg11* in our studies, however. Our results suggest that *Spg11* loss of function contributes to neurodegeneration primarily through immune dysregulation and lipid imbalance, rather than affecting α -synuclein processing pathways.

While we found no evidence linking *Spg11* loss of function and α -synuclein aggregation, previous publications have reported that α -synuclein aggregates more readily in a high-lipid environment and we did observe changes in lipid processing in the midbrain. Indeed, the α -synuclein A53T mutant we used here was found by Flagmeier, et al. to have the highest rate of lipid-induced aggregation of any of the isoforms studied [46]. It is possible that the immunohistochemical methods we used to measure α -synuclein pathology may not have detected more subtle changes if they were present. It is also worth noting that α -synuclein is primarily found in neurons, while our in vitro studies focused on myeloid cells, as these are the cell types we found to be most affected by loss of spatacsin in transcriptomic analysis. Interestingly, while the presence of α -synuclein aggregates was found to increase the levels of oxidative cholesterol in cells, we did not find that expression of A53T α -synuclein affected lipid accumulation in BMDMs [47]. Thus, while we found no link between SPG11 and α -synuclein pathology, it remains unclear why these two seemingly related pathways do not intersect and what distinguishes the biology of the lipid abnormalities in these two conditions.

Microglial activation in hereditary spastic paraplegia

In our studies, we found that loss of *Spg11* induces a pro-inflammatory profile in microglia in the midbrain. While this appears to be an α -synuclein-independent process, it should be noted that microglia also exhibit increased reactivity in PD. As neurodegeneration progresses, the density of activated microglia in the nigrostriatal pathway increases as dopaminergic neurons degenerate [48]. Indeed, it is thought that microglia in the PD brain may exhibit impaired phagocytic capabilities, perhaps linked to dysfunctional lysosomes observed in disease pathogenesis [49]. While the inflammatory profile induced in microglia by loss of function did not alter α -synuclein aggregation in either of our synucleinopathy models, it is interesting that both disorders share this characteristic inflammatory profile of the midbrain. Future studies may determine how the activation of microglia differs between HSP and other diseases with parkinsonism features to shed insights into common and contrasting aspects of neuroinflammation in both diseases.

Inflammation in the brain of *Spg11*^{-/-} mice has been previously described, although not in the midbrain, which was the region this study focuses on [33, 34, 50]. A study of *Spg11*^{-/-} mice, primary microglia and BMDMs from those animals, and human monocyte-derived

macrophages from SPG11-HSP patients demonstrated increased immune reactivity mediated by the non-canonical inflammasome pathway with loss of *SPG11* function. The authors of this paper demonstrate that this is a cell-autonomous pathway and that increased immune reactivity precedes neurodegeneration [51]. Interestingly, a similar mouse model of HSP using *Spg15* knockout also observed neuroinflammation, and they concluded that this inflammation was preceding neuronal loss in that model [52]. Additionally, both the *Spg11*^{-/-} and *Spg15*^{-/-} models have reported T-cell infiltration, and follow-ups to determine if this is a necessary step in the observed neuron loss and inflammation or a byproduct of existing microglia signaling [34, 50].

Lipid accumulation and gliosis had both been previously reported to occur in the CNS of animals lacking functional *Spg11* and in patients with SPG11-HSP [18, 20, 34]. Our study demonstrated that this lipid accumulation happens in the microglia themselves and appears to be cell intrinsic. Previous reports around lipid accumulation have focused on lipids building up in neurons specifically, going so far as to demonstrate that inhibiting the synthesis of gangliosides, the lipid species found to increase in the lysosomes of *Spg11*^{-/-} neurons, decreased neuronal vulnerability [25]. Microglia and myeloid cell activation were previously reported in *Spg11*^{-/-} animals and in patient-derived iMicroglia cells [33, 34]. In this study, we showed that microglia are accumulating lipids in the brains of *Spg11*^{-/-} mice, supporting the hypothesis that microglia are more prone to activation due to toxic lipid buildup. Our findings position microglial lipid metabolism as a potential therapeutic target in *SPG11*-related disease. Future studies should test whether pharmacological or genetic strategies that restore lipid homeostasis in microglia can reduce neuroinflammation and slow neurodegeneration in HSP and related disorders.

Materials and methods

Mouse models

All animal studies were approved by Genentech's Institutional Animal Care and Use Committee, in accordance with guidelines that adhere to and exceed state and national ethical regulations for animal care and use in research. SPG11^{-/-} mice were obtained from Frédéric Darios, having been created by inserting early stop codons in exon 32 [20]. A53T mice express human α -synuclein with an A53T mutation under the mouse prion protein promoter and were derived from Jackson Laboratories, Bar Harbor, ME, USA (stock #004479) [39]. For animals without A53T expression, heterozygous animals were bred to produce littermate controls. A hemizygous breeding scheme was used to produce animals with both *Spg11*^{-/-} mutations and A53T expression. Male and female mice were used in all studies.

Mice were housed on a regular light/dark cycle (14:10 h) with *ad libitum* access to food (LabDiet 5010) and water. All behavioral assessments were conducted during the light phase. Mice received DietGel 76 A (Phar-maServ) for a week following substantia nigra injection surgery.

All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Substantia nigra injections

Animals were injected with human α -synuclein PFFs in similar manner as to what has been previously described [37, 53]. Animals were anesthetized with isoflurane in an induction chamber and transferred to a stereotaxic frame (Stoelting Co.) where they were given continuous isoflu-rane via nose cone. Body temperature was maintained during the surgery using a heating pad and ophthalmic ointment was applied to both eyes of the animal prior to initiation of surgery. Following alignment on the frame, a midline incision was made along the scalp and opened to identify bregma. Using a microdrill with a burr tip size of 0.5 mm in diameter, a small hole was drilled through the skull above the injection site. The following stereotaxic coordinates were used to inject into the substantia nigra: anterior–posterior $\pm$ 3.0 mm, medio-lateral $\pm$ 1.5 mm, dorso-ventral $\pm$ 4.6 from bregma. At these coordinates, 5 μ l of PFF (Stressmarq, Cat # SPR 317) at 2 mg/mL or sterile saline was injected using a 26 gauge 10 μ l Hamil-ton syringe. The injection was slowly infused at a rate of 0.25 μ l/min and the needle was kept in place for at least 3 min post-injection to prevent backflow. Finally, the incision was closed with tissue adhesive (vetbond) and animals were allowed to recover with meloxicam admin-istered for pain relief.

Hangtime assay

Mice were enrolled in behavior assays starting at 40 weeks old as previously described.⁵⁴ They received 3 rounds of training on hangtime, then were assayed at the designated times thereafter. To perform hangtime mice were placed on a wire grate, which was gently inverted after they had grasped it. The mouse was allowed 3 tries to hang for a maximum of 90 s. All scoring was done blind to genotype.

Immunohistochemistry, imaging, and analysis

Animals were deeply anesthetized and transcardially per-fused with cold PBS then 4% paraformaldehyde in PBS. Brains were removed, drop-fixed in 4% paraformalde-hyde for 24 h at 4 °C, then transferred to PBS and shipped to NeuroScience Associates, Knoxville, TN, USA for sec-tioning and staining. Brains were multiply embedded in

a gelatin matrix using MultiBrain Technology (NeuroSci-ence Associates) and coronally sectioned at 30 μ m.

Immunohistochemistry was performed using rab-bit anti-Iba1 (Wako, 019-19741), mouse anti-CD68 (Biorad, MCA1957), rabbit anti-TH (Pel-Freez Bio-logicals, P40101), rabbit anti-pSer129 α -synuclein (abcam, ab51253), and rat anti-Clec7a (invivogen, mabg-mdect-2). Aminocupric silver stain was performed by NeuroScience Associates. Stained brain tissues were images using a Hamamatsu Corp Nanozoomer at 20X. At least 5 sections per animal were analyzed.

Substantia nigra regions were identified using the pro-gram described in Barzekar et al., 2023. Regions were verified by manual visualization and corrected when nec-essary [54, 55]. Regions of the cortex and midbrain were manually drawn. Analysis of immunohistochemical stain-ing was done by using color thresholds and morphologi-cal operations. Cell segmentation was performed using Cellpose with training as described in Stringer et al., 2022 [55]. Analysis of staining was done by using color thresh-olds and morphological operations. A regional minima and radial symmetry detection with watershed segmen-tation followed by morphological, shape, and size filter-ing were used to determine positive cells [56]. Microglia ramification was calculated using a previously described morphometric analysis of segmented microglia cells in tissue [35]. Image analysis was performed blinded to experimental grouping and genotype.

RNA extraction sequencing and quality control

Total RNA was isolated from the midbrain of 5 SPG11^{-/-} mice and 5 SPG11^{+/+} littermate control brain tissue using the RNeasy Universal Mini Plus Kit (Qiagen) follow-ing the manufacturer's instructions. Briefly, tissue was homogenized in QIAzol lysis reagent with a TissueLyser II (Qiagen), and phase separation was performed with chloroform. The aqueous phase was mixed with etha-nol and applied to RNeasy spin columns for RNA bind-ing, followed by on-column genomic DNA elimination and sequential washes with kit buffers. RNA was eluted in RNase-free water, quantified by NanoDrop spectro-photometry (Thermo Fisher Scientific), and integrity was assessed using an Agilent Bioanalyzer 2100.

RNA-seq libraries were prepared from 0.15–1 μ g of total RNA per sample using the *TruSeq* RNA Sample Preparation Kit v2 (Illumina). Single-end, 50 bp reads were generated on an Illumina HiSeq sequencer, aim-ing for approximately 30 million reads per sample. Raw sequencing reads were subjected to quality control using FastQC (version 0.12.0) to assess read quality, identify potential adaptor contamination, and evaluate sequence biases. Results were then aggregated using MultiQC (v1.9) [57]. Based on FastQC reports, adapter sequences and low-quality bases were trimmed from the 3' end of

reads using fastp (v0.23.3) with default parameters [58, 59]. Post-trimming quality control was performed to ensure sufficient read quality for downstream analysis.

Read alignment and gene quantification

Trimmed reads were aligned to the mouse reference genome GRCm38 using HTSeqGenie (version 3.20) [60]. Reads were aligned, and gene-level counts were quantified based on “Ensembl GRCm38.97 annotations from BioMart [61].

Differential expression analysis

Differential gene expression between SPG11^{-/-} and wild-type animals was performed using the limma-voom package (version 3.61.12) in R (version 4.4.0) [62, 63]. Raw gene counts were normalized using the trimmed mean of M-values (TMM) method and the ‘voom’ function was applied to transform the RNA-seq count data to log2 counts per million (log2 CPMs) with associated precision weights, which account for the mean-variance relationship inherent in RNA-seq data. A linear model was then fitted for each gene, and empirical Bayes moderation was used to estimate gene-wise variances. Differential expression was determined using a contrast comparing SPG11^{-/-} to SPG11^{+/+}. Genes with an adjusted p-value (Benjamini-Hochberg false discovery rate, FDR) < 0.05 and a |log2 fold change| > 1 were considered statistically significant.

Heatmap visualization

A heatmap visualizing the expression of differentially expressed genes was generated using log2 CPM values. To highlight relative expression changes across samples, the log2 CPMs were Z-score normalized for each gene before heatmap generation. The heatmap was visualized using the ComplexHeatmap (v2.21.1) package in R [64].

A separate heatmap was generated to specifically visualize the expression of genes associated with the Gene Ontology (GO) term GO:0033993: “Response to lipid.” Log2 CPM values were used, and Z-scores were calculated for each gene within this subset for visualization purposes.

GO term enrichment analysis

Pathway enrichment analysis was performed on the full set of differentially expressed genes using the ClusterProfiler (v4.13.4) package in R [65]. Gene set enrichment analysis (GSEA) was conducted using the ‘fgsea’ method, which is implemented within ClusterProfiler, to identify biological pathways that were significantly enriched in SPG11^{-/-} animals compared to wild-type [66]. This analysis specifically employed Gene Ontology Biological pathways. Pathways with an adjusted p-value (FDR) < 0.05 and |NES| > 1 were considered significant.

Comparison to Allen Brain Atlas Single-Cell Data

To investigate the cell type specificity of the observed transcriptional changes, a comparative analysis was performed using publicly available single-cell RNA sequencing data from the Allen Brain Atlas (<https://mouse.brain-map.org/>). Pseudobulk counts were generated for each major cell type present in the Allen Brain Atlas dataset. For each cell type, a “score” for the differentially expressed genes (between SPG11^{-/-} and WT) was calculated by determining the average expression of these genes within that specific cell type’s pseudobulk counts for each sample. This approach allowed for an estimation of the relative enrichment of the SPG11^{-/-}/WT differential expression signature across various brain cell types.

Flow cytometry

The unfixed hemibrain isolated from mice was carefully dissected, mechanically dissociated, and enzymatically digested at 37 °C for 1 h with agitation. The digestion buffer consisted of collagenase D (Sigma, 50 mg/ml), TLCK trypsin inhibitor (Sigma, 100 µg/ml), DNase I (Sigma, 100 U/µl), HEPES buffer (1 M, pH 7.2; Gibco), prepared in HBSS (Gibco). Following digestion, the tissue suspension was passed through a 70 µm cell strainer and centrifuged at 500 × g for 10 min. The resulting cell pellet was resuspended in 37% Percoll and centrifuged at 1,200 × g for 30 min to remove myelin debris. Cells were then washed and resuspended in FACS buffer.

For antibody staining, cells were first incubated with TruStain FcX for 10 min at 4 °C to block Fc receptors. This was followed by surface staining with fluorescently labeled antibodies for 20 min at 4 °C, and subsequent fixation and permeabilization for 30 min at 4 °C. Cells were then stained intracellularly for 45 min at room temperature. Samples were resuspended in FACS buffer and acquired using a BD LSRFortessa X-20 flow cytometer. Data were analyzed with FlowJo software.

Primary culture of bone marrow-derived macrophages

Bone marrow was extracted from the femurs and tibias of adult mice between the ages of 2 and 4 months, by cutting the ends of bone and removing the marrow through centrifugation. The bones were placed cut end down in a 0.6 mL eppendorf tube that had been punctured with a needle and that 0.6 mL tube was placed in 1.5 mL eppendorf tube, then spun at max speed for 1 min. Marrow was resuspended in 40 mL DMEM media with 10% fetal bovine serum (xxx) and 1% penicillin-streptomycin (Gibco) supplemented with xxx mouse CSF (Biotechne). Resuspended cells were split between two 15 cm non-treated dishes (Falcon). Cells were allowed to proliferate for 5 days, with 10 mL of media added to each place on day 2. On the 5th day of culture, cells were washed 3 times with PBS to remove red blood cells, then gently lifted with a cell scraper in about 10 mL of media. Cells were spun for 5 min at 7,000 g then the pellet was

resuspended in media (same as described above) and plated into 96 well plates.

Phagocytosis imaging and analysis

Phagocytosis assay was performed on primary BMDMs similarly to what has been previously described [67]. BMDMs were plated in 96-well plates at 300,000 cells/mL. 24 h after plating, pHrodo-Red zymosan particles (Invitrogen, P35364) were added to the culture according to manufacturer protocol. Images were taken using an IncuCyte S3 instrument and software every hour for 16 h. A custom analysis script within the IncuCyte S3 software was used to measure the phagocytosed particles and the confluency of cells. Cells positive for having phagocytosed the particles were normalized to the total confluency of the well. The amount SPG11^{+/+};A53T.neg normalized phagocytosis at 16 h was set to 100% and all other genotypes were normalized to this scale.

Lipid droplet imaging and analysis

Primary BMDMs were cultured in 96-well plates (Corning) at 200,000 cells/mL for 24 h. One day after plating, cells were treated for 24 h with PBS or with oleic acid at 100 μ M (Sigma-Aldrich, O3008). BMDMs were fixed for 10 min in 4% PFA, washed 3 times with PBS, incubated for 10 min in 1:1000 488 Lipidspot (Biotium) and 1:1000 Hoechst, then rinsed with PBS. Lipid droplet (LD) imaging was performed using an ImageXpress Confocal HT.ai High-Content Imaging System (Molecular Devices) equipped with an 89 North LDI illuminator. Images were acquired with a 40X APO LWD 1.15 NA WI objective (Nikon) and analyzed using the Custom Module Editor in MetaXpress v6.7.2.290 (Molecular Devices). Adaptive background subtraction was applied, and LDs as well as nuclei (DAPI-positive puncta) were thresholded. The total number of LD puncta was quantified and normalized to the total number of nuclei per image. Data were plotted using GraphPad Prism.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12974-026-03910-3>.

Supplementary Material 1.

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Authors' contributions

M.H. designed and performed experiments, analyzed data, and wrote the manuscript. J.L. designed and performed experiments, analyzed data, and edited the manuscript. T.L. and A.S. performed experiments and analyzed results. M.A. analyzed data. S.G., H.L., S.C., K.S. performed experiments. H.N.

analyzed data. O.F., J.K., and J.H. supervised the research. C.C.H. supervised the research, coordinated the study, and wrote the manuscript.

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Data availability

The RNA-seq datasets generated in this paper are available at GEO: GSE324086. Reagents generated in this study are available upon request.

Declarations

Ethics approval and consent to participate

All animal studies were approved by Genentech's Institutional Animal Care and Use Committee, in accordance with guidelines that adhere to and exceed state and national ethical regulations for animal care and use in research.

Consent for publication

Not applicable, no human subjects were used in this study.

Competing interests

All authors are employees of Genentech, Inc., a member of the Roche group. The authors declare that they have no additional conflict of interest.

Author details

¹Department of Neuroscience, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA

²gRED Computational Sciences, Genentech, Inc., South San Francisco, CA 94080, USA

³Department of Research Pathology, Genentech, Inc., South San Francisco, CA 94080, USA

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