# Alpha-synuclein supports type 1 interferon signalling in neurons and brain tissue

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## Abstract

The protein α-synuclein (αSyn) is predominantly expressed in neurons and is associated with neurodegenerative diseases like Parkinson's disease (PD) and dementia with Lewy bodies. However, the normal function of αSyn in neurons is not clearly defined. We have previously shown that mice lacking αSyn expression in the brain exhibit markedly increased viral growth in the brain, increased mortality, and increased neuronal cell death implicating αSyn in the neuronal innate immune response. To investigate the mechanism of αSyn-induced immune responses to viral infections in the brain, we challenged αSyn knock-out (KO) mice and human αSyn KO dopaminergic neurons with RNA virus infection and discovered that αSyn is required for neuronal expression of interferon-stimulated genes (ISGs). Furthermore, human αSyn KO neurons treated with type I interferon (T1IFN) failed to induce a broad range of ISGs implying that αSyn interacts with T1IFN signaling. We show that total and phospho-serine129 αSyn (pS129 αSyn) accumulates in the nucleus of T1IFN-treated human neurons and human brain tissue in patients with viral encephalitis. We also demonstrate that T1IFN-mediated phosphorylation of STAT2 is dependent on αSyn expression in human neurons and that phosphorylated Stat2 co-localizes with αSyn following T1IFN stimulation in neurons. Taken together, our results show that αSyn supports neuron-specific T1IFN responses by supporting STAT2 activation, localizing to the nucleus, co-localizing with phosphorylated Stat2 in neurons, and supporting expression of ISGs. These data also provide a novel mechanism by which T1IFN

stimulation triggers expression of pS129 αSyn, a post-translational modification of αSyn associated with PD pathogenesis.

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Abbreviations: αSyn=alpha-synuclein; ISG=interferon stimulated gene; KO=knockout; MFI=mean fluorescence intensity; PD=Parkinson's disease; T1IFN=type 1 interferon; VEEV=Venezuelan Equine Encephalitis virus; WEEV=Western equine encephalitis virus; WNV=West Nile virus; WT=wild-type

# Introduction

Alpha-synuclein (αSyn), a 14 kDa protein encoded by the *SNCA* gene, is best known as the primary component of Lewy bodies found in patients with Parkinson's disease (PD) as well as other synucleinopathies[1]. αSyn is predominately expressed in neurons both in the central and peripheral nervous systems as well as red blood cells[2,3]. Previous studies exploring a functional role for αSyn expression in neurons have suggested a variety of potential cellular functions. Most notably, studies have suggested a potential role for αSyn in regulating synaptic transmission and vesicle transport. *Snca*$^{-/-}$ mice show an increased release of dopamine with paired stimuli and a corresponding reduction in striatal dopamine levels[4]. Basal dopamine levels were also shown to be reduced in the absence of both alpha-synuclein and beta-synuclein, though not in single gene knockout animals[5]. Additionally, αSyn deficient mice have impaired hippocampal synaptic responses to prolonged stimuli that are associated with a deficiency in undocked vesicles in the synapse[6]. This function of αSyn in regulating synaptic transmission and vesicle transport may be tied to an association between αSyn and SNARE-proteins[7]. In addition to this potential native function of αSyn, recent evidence suggests a role for this protein in facilitating innate immune responses in the CNS.

Numerous studies suggest αSyn modulates innate immune responses in the CNS. We previously reported that αSyn knockout (KO) mice (*Snca*$^{-/-}$) exhibit increased mortality and disease severity from viral encephalitis, accompanied by increased viral growth in the brain following peripheral challenge with RNA viruses including West Nile virus (WNV) and Venezuelan equine encephalitis virus (VEEV) TC83[8]. An important subsequent study demonstrated that total αSyn expression was elevated in gastrointestinal-associated neurons following viral gastroenteritis in children[9]. The same study also suggested that αSyn expression

supported chemotaxis and activation of infiltrating dendritic cells[9]. A more recent follow-up study has shown that αSyn is a critical mediator of inflammatory and immune responses in gastrointestinal tract by supporting T-cell responses.[10] αSyn was additionally shown to be necessary for controlling intranasal infection with reovirus and intravenous infection with *Salmonella typhimurium*[11]. Another study was able to show that intranasal inoculation of Western equine encephalitis virus (WEEV) was shown to result in loss of dopaminergic neurons in the substantia nigra and formation of prominent proteinase K-resistant aggregates of phospho-serine129 αSyn (pS129 αSyn).[12] Taken together, these studies have shown that acute infection increases αSyn expression in mice, increases phosphorylation of αSyn at serine residue 129, and can result in loss of nigral dopamine neurons; thereby recapitulating multiple key neuropathological features of PD. Despite these important recent findings, the mechanisms underlying the functional role of αSyn in the immune response in the CNS remain unclear.

In the current study, we performed RNA-Seq analysis on whole brain tissue from wild-type (WT) and αSyn KO mice infected with WNV to determine differences in gene expression that may contribute to the deficient immune response in αSyn KO mice. We found that αSyn KO mice exhibited deficiencies in the expression of several interferon stimulated genes (ISGs) that are critical for the control of viral infection in the CNS. To investigate where αSyn is acting in its immune function, we next used human dopaminergic neurons differentiated from WT and αSyn KO human embryonic stem cells[13]. We found that αSyn KO neurons challenged with VEEV TC83 exhibited significantly increased viral growth. Following stimulation of human and mouse neurons with type 1 interferon, we found that αSyn localizes to the nucleus, co-localizes with phosphorylated STAT2, that STAT2 phosphorylation is dependent on αSyn expression and that ISG expression is significantly reduced in the absence of αSyn. In human brain tissue, we

also show that patients with West Nile virus encephalitis exhibit increased pS129 αSyn expression with increased pS129 αSyn localization to the nucleus. Thus, our data are the first to demonstrate that αSyn functions to modulate interferon signaling in human neurons and brain tissue by supporting STAT2 activation and downstream interferon stimulated gene expression.

# Materials and Methods

## Resource availability

### Lead contact

Further information and requests for reagents may be directed to and be fulfilled by the corresponding author, Dr. J. David Beckham (david.beckham@cuanschutz.edu).

### Materials availability

Experimental models (organisms, strains) generated for use in this study will be made available on request, but we may require a completed Materials Transfer Agreement if there is potential for commercial application. These materials are available for distribution under the Uniform Biological Material Transfer Agreement, a master agreement that was developed by the NIH to simplify transfers of biological research materials.

### Data and code availability

The published article includes all datasets generated or analyzed during this study. Please see gene expression data for full list of genes analyzed using RNAseq of brain tissue (**Supplemental**

**Table 1**). Groups A, B, C, and D represent mock-infected WT, mock-infected αSyn KO, WNV-infected WT, and WNV-infected αSyn KO mice, respectively.

## Experimental model and subject details

### Ethics statement

All animal work was performed at the University of Colorado Anschutz Medical Campus in accordance to and following approval by the Institutional Animal Care and Use Committee. All work with live viruses and recombinant DNA was approved by the University of Colorado Institutional Biosafety Committee and performed in accordance with local and national regulations of pathogens. Human brain tissue was obtained from de-identified human autopsies at the University of Colorado Hospital with approval for non-human research by the local Colorado Multiple Institutional Review Board. All work with hESCs was completed at the University of Edinburgh and ethics approval was granted by the MRC Steering Committee for the UK Stem Cell Bank and for the Use of Stem Cell Lines (ref. SCSC13-19).

### Cell culture

All cell lines were maintained at 37°C in 5% $CO_2$. *SNCA*$^{-/-}$ and *SNCA*$^{+/+}$ human embryonic stem cells (hESCs) were generated and differentiated towards midbrain dopaminergic neural progenitors to day 16 by the Kunath laboratory as previously described[13]. Following this, the cells were cryopreserved as described[14] and shipped to the Beckham laboratory. Cells were then thawed and plated in Laminin-111(BioLamina)-coated 48-well plates at a density of 800,000 cells/cm$^2$ in neuronal differentiation media consisting of Neurobasal Media (Thermo Fisher Scientific) + B27 supplement (without Vitamin A, 1:50, Thermo Fisher Scientific) + l-Glutamine (2 mM, Thermo

Fisher Scientific) supplemented with ascorbic acid (AA, 0.2 mM, Sigma), brain-derived neurotrophic factor (BDNF, 20 ng/ml, Peprotech), glial cell line-derived neurotrophic factor (GDNF, 10 ng/ml, Peprotech), dibutyryl cyclic AMP (dbcAMP, 0.5 mM, Sigma), and DAPT (1 μM, Tocris). Y27632 (Y2, 10 μM, Tocris) was present in medium from day 16 to day 17. Cells were differentiated for an additional 26 days (42 days of total differentiation), and media was replenished every 3-4 days. RC17 hESCs were differentiated into cortical neurons using a dual-SMAD inhibition protocol.[15] RC17 hESCs were expanded on 5 μg/mL laminin-521 (Biolamina LN521-05) in iPS-Brew (Miltenyi Biotech) before being replated in 24-well plates coated with 5 μg/mL laminin-111 (Biolamina LN111-04) and fed every 2 days with differentiation media (1x N2, 1x B27, Neurobasal, DMEM:F12 w/ l-glutamine), with days 0 – 4 of cortical medium supplemented with 10 uM SB431542 (Merck 616461) and 100 nM LDN (Miltenyi Biotech 130-103-925). Cells were replated on days 11, 16 and 25 into 8-channel μ-Slides (Ibidi 80801) coated with 15 μg/mL poly-l-orithine (Sigma P4957) and 5 μg/mL laminin-111 at a density of 100,000 cells per $cm^2$ and fed twice-weekly with differentiation media containing 20 ng/mL BDNF and 10 ng/mL GDNF (Peprotech, 450-02-100 and 450-10-100). Cells were further differentiated until day 40. Cortical identity was determined with immunostaining of Pax6+ cells at days 11 and 16, and with Tbr1+ and Ctip2+ staining at day 55. Murine primary cortical neurons were harvested from E18 C57B6 embryos as previously described.[16] Murine primary cortical neurons are differentiated in serum-free Neurobasal media (Thermofischer) for 7 days prior to use in experiments and are verified to be 98% neurons using immune fluorescence quantification with antibodies specific for Map2 (neuron marker), Iba1 (microglia marker), and Gfap (astrocyte marker) prior to use.

**Virus propagation and quantification**

West Nile virus strain 385-99 (NY99) was obtained from clone derived virus and propagated in Aedes albopictus (C6/36, ATCC CRL-1660) cells as previously described.[8] Venezuelan Equine Encephalitis virus (VEEV) TC83 isolates were obtained from the laboratory of Dr. Michael Diamond at Washington University in St. Louis and was propagated in BHK cells. Viral titers for all viruses were quantified in Vero cells by standard plaque assay as previously described[8]. Viral genome was quantified by probe-based qRT PCR.

The 3'UTR of WNV was amplified using forward primer CAG ACC ACG CTA CGG CG, reverse primer CTA GGG CCG CGT GGG, and probe /6FAM/TCT GCG GAG AGT GCA GTC TGC GAT/MGBNFQ/. The 15 nsP1 gene of VEEV TC83 was amplified using the forward primer GCC TGT ATG GGA AGC CTT CA, reverse primer TCT GTC ACT TTG CAG CAC AAG AAT, and probe 6-FAM/ CCT CGC GGT /ZEN/ GCA TCG TAG CAG C/ 3IABkFQ/. Quantification was achieved by generating standard curves of serial diluted plasmids of known copy number and normalized to 18s rRNA copies. For 18s rRNA quantification, priming was achieved with the forward primer CGC CGC TAG AGG TGA AAT TC, reverse primer sequence CAT TCT TGG CAA ATG CTT TCG, and probe /6-FAM/CAA GAC GGA CCA GAG CGA AAG CAT/TAMRA/.

**Mouse Studies**

*Snca*$^{-/-}$ mice were obtained from Jackson Laboratories (#3692) and back-crossed seven generations to C57BL/6J mice (#664). Microsatellite analysis performed by Jackson Laboratories confirmed mice were 96.3% C57BL/6J. These mice were crossed with WT C57BL/6J mice to generate *Snca*$^{+/-}$ heterozygous mice. Genotyping by conventional PCR was routinely performed to confirm *Snca* status as described[8]. Virus used for infections was first

diluted to the appropriate viral titer in HBSS before being administered by subcutaneous injection, intracranial injection, or corneal inoculation.

Prior to subcutaneous inoculation of virus, all mice were randomized, weighed, and placed under isoflurane-induced anesthesia. Equal numbers of male and female mice were used for all studies with the exception of the use of only female mice for the total brain RNAseq analysis. For subcutaneous injections, 10 uL of virus solution was injected into the left footpad of mice with the use of a Hamilton syringe. For intracranial injections, 10 µL of virus solution was injected 3 mm deep into the brain near the Bregma with the use of a 25 gauge needle. Mice were monitored for morbidity and weighed daily. Mice losing more than 15% bodyweight prior to the end of the study were euthanized and excluded from the study for humane reasons.

At the end of each experiment, mice were euthanized by isoflurane overdose before proceeding with tissue harvest. All mice were perfused with 20 mL of phosphate buffered saline solution (PBS) prior to tissue harvest. Samples collected for RNA gene expression assays or viral quantification were collected and stored in RNALater (Invitrogen, #AM7021) or PBS, respectively. Samples stored in RNALater and PBS were stored at -80 C.

**Neuronal Cell Culture Infections and Interferon Treatments**

*SNCA*$^{-/-}$ and *SNCA*$^{+/+}$ hESC-derived midbrain dopaminergic and cortical neurons were grown as described above. Following this, the cellular media was removed and replaced with complete neuronal differentiation media (described above) containing VEEV-TC83 virus at a multiplicity of infection (MOI) of 1. 300 µl of the media was removed and replaced with fresh, virus-free neuronal differentiation media every 12 hours for 72 hours. The viral content of these samples was then titered via plaque assay as described previously[8]. Differentiated 15 neurons were

infected with VEEV-TC83 at an MOI of 10. 12 hours post-infection, RNA from these cells was extracted and analyzed via qPCR (described below). Differentiated neurons were treated with 10,000 IU/mL of mixed-type human IFNa, 25 μg/mL of Poly(I:C), or mock treated. RNA from these cells was extracted 4 hours post-treatment and analyzed via qPCR and protein studies were completed at the indicated time points.

**Gene expression assays**

RNAs collected from tissue were extracted using Trizol-chloroform extraction followed by column based isolation using the E.Z.N.A Total RNA kit II (Omega Bio-Tek #R6934) according to manufacturer's protocol. RNAs collected from cells were extracted using E.Z.N.A Total RNA kit I (Omega Bio-Tek #R6834) according to manufacturer's protocol. Qiagen's custom $RT^2$ Profiler PCR array was used to assay isolated microglia and neurons from infected mouse brain for interferon stimulated genes and microglia activation markers. RNA extraction and purification, cDNA synthesis, and Qiagen $RT^2$ custom PCR array was performed according to manufacturer's protocol and recommendations.

BioRad's PrimePCR probe-based qPCR assays for *Oas1b*, *Irf9*, *Tlr3*, *Trim25*, *Tgtp1*, and *Mx1* were used to verify RNAseq results. RNA extraction, cDNA synthesis, and qPCR was performed according to manufacturer's protocols and recommendations. Normalization was achieved using 18s rRNA copy number as described above. BioRad's PrimePCR SYBR Green-based qPCR assays for*TRIM25* and *IFIT1* were used to analyze gene expression during infection and following immune-pathway activator treatment (e.g. Poly I:C) of differentiated $SNCA^{-/-}$ and $SNCA^{+/+}$ hESC-derived neurons. RNA extraction, cDNA synthesis, and qPCR were performed according to manufacturer's protocols and recommendations. Normalization was achieved by calculating the ΔCT value of each sample compared to expression of the housekeeping gene

*GAPDH*. Relative expression was calculated by comparing the ΔCT value of each sample to the average ΔCT value of the mock infected, WT mouse samples.

RNAseq analysis was performed on bulk brain RNAs by Novogene (Sacramento, CA, USA). Analysis of sequencing reads completed by Novogene and additional analysis of Fastq files completed in the Beckham laboratory. Following total RNA quantification (Nanodrop) from brain tissue, we completed mRNA enrichment (poly-T oligo-attached magnetic beads), cDNA synthesis, end repair, poly-A and adaptor addition, fragment selection and PCR, and library quality assessment (Agilent2100) followed by Illumina sequencing (NovaSeq). Following data clean up, clean reads representing 96.51% of total reads were available for analysis at 5 approximately 60-80million reads per sample. Mouse genome sequence alignment was completed with STAR with 85.7% of reads mapping to exons and fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) calculated for gene expression. Overall gene expression was analyzed using principal component analysis and DESeq2 R package for differential expression analysis. ClusterProfiler software was used for enrichment analysis including GO enrichment, DO enrichment, KEGG and Reactome database enrichment.

**ELISA Assays**

Protein samples were acquired from *SNCA*$^{-/-}$ and *SNCA*$^{+/+}$ hESC derived neurons during infection and following immune-pathway activator treatment (e.g., Poly (I:C)) using TRIzol purification (Invitrogen #15596026) according to the published protocol (Pub. No. MAN0001271). Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Following this, concentrations of STAT1 and P-STAT1 were determined using the Human Phospho-STAT2 (Tyr689) and Total STAT2 ELISA Kit (RayBiotech).

## Immunocyotchemistry

Differentiated hESC derived neurons and murine primary neurons were grown on Laminin-111 treated coverslips placed inside 24 well plates as described above. Neurons were then treated with IFNa2 for 1 or 4 hours at a concentration of 10,000 IU/ml. Following this, cells were fixed in 4% paraformaldehyde for 10 minutes and washed 3 times with D-PBS containing calcium and magnesium. Cells were stored in D-PBS containing calcium and magnesium for 3 days prior to staining at 4°C. Cells were then permeabilized in 0.3% Triton-x in D-PBS for 5 minutes at room temperature. Permeabilized cells were then rinsed 3 times in cold D-PBS. The cells were then incubated in 5% goat serum in D-PBS containing calcium and magnesium for 1 hour at room temperature. Following this, the serum solution was removed, and cells were incubated in a 5% goat serum solution in D-PBS containing calcium and magnesium containing a 1:300 dilution of mouse anti-human alpha-synuclein antibody conjugated to FITC (Novus) and a 1:200 dilution of rabbit anti-human PSTAT2 antibody (Invitrogen) overnight at 4°C. The primary antibody solution was then removed and the cells were washed 3 times in D-PBS containing calcium and magnesium. Cells were then incubated in a 5% goat serum solution in D-PBS containing a 1:200 dilution of goat anti-rabbit IgG conjugated to TRITC (Jackson ImmunoResearch) for 1 hour at room temperature. Cells were then rinsed 3 times in D-PBS with calcium and magnesium. The coverslips containing the cells were then removed and affixed to microscopy slides using ProLong Gold Antifade Mountant with DAPI (Invitrogen). Cells were subsequently imaged at 60x magnification using an Olympus FV1000 Confocal Laser Scanning Biological Microscope (Olympus). Cortical neurons treated and fixed were blocked with 2% goat serum for 30 mins, permeabilized with 0.1% Triton X-100 and stained for total alpha-synuclein (BD Biosciences 610787) and beta-III Tubulin (R and D Systems MAB1195) overnight, then stained for secondary antibodies Alexa 488

(Thermofisher A21121) and Alexa 568 (Thermofisher A21134) for 1 hr, before staining with DAPI. Imaging was conducted with Leica SP8 confocal imaging at 63x magnification. Images were randomized, blinded, and given to a separate researcher for qualitative analysis of colocalization (FIJI Co-loc, Manders analysis scoring). For cortical neurons, Images were processed using ImageJ macro (w/ StarDist plugin, courtesy of Dr Matthieu Vermeren) to quantify alpha-synuclein staining in DAPI space as a measure of nuclear localisation. Quantified image data was processed using R Studio script with a threshold of 4132 MFI (mean fluorescence intensity) and 10 $\mu m^2$ DAPI-stained area set as a distinguisher of nuclear localization in viable cells, whereby the threshold was used to calculate percentage of nuclear-localized nuclei per image. An unpaired t-test was used to measure statistical significance between treatment groups. The threshold was determined by calculating a cumulative frequency distribution of MFI of all nuclei in both treatment groups. 4132 MFI was chosen as the nuclear localization threshold using a cumulative frequency distribution analysis (PRISM 9) to exclude basal level nuclear localization, whereby the highest MFI of either group with a 75% percentile of the nuclear population (IFNA2) and 89% percentile of the total nuclear population had an MFI below this value.

**Immunohistochemistry of Human Tissue**

Unstained slides prepared from formalin fixed, paraffin embedded tissue were obtained from various brain regions from all three cases. Slides from midbrain, basal ganglia, thalamus, pons, frontal cortex, and superior cerebellum were obtained from the WNV positive case. Tissue from the midbrain of control case 1 and thalamus of control case 2 were obtained. Renal tissue slides were obtained from the WNV negative case to serve as a negative control to determine the degree of non-specific antibody binding and autofluorescence. A positive control midbrain slide

from a patient with Parkinson's disease and increased phospho-S129 αSyn (pS129 αSyn ) expression was used as a positive control.

Blank slides were deparaffinized using Bioss protocol. Antigen retrieval was performed via HIRS method using Vector Antigen Unmasking stock solution diluted 100x (Vector). Slides were submerged in the buffer and microwaved in a pressure cooker for 14 minutes to achieve a temperature of 100C, then left to stand for an additional 1 minute. Slides were washed in DI to bring them to room temperature while preventing tissue dehydration. Slides were then blocked with Blocking Buffer(TSBB, Thermo Scientific) for 30 minutes at room temperature. Blocking buffer was drained off the slides. PS129 αSyn (Abcam EP1536Y) and FOX3(Biolegend 1B7) primary antibodies were diluted in TSBB at a concentration of x and y respectively and applied to slides at 400uL per slide. Slides were allowed to incubate overnight at 4ºC, then rinsed in TBST for 5 minutes, 2 times. TRITC and FITC fluorescent labeled secondary antibodies were diluted in TBSS at concentrations of 1:100 and applied to slides at 400uL per slide and allowed to incubate for 2 hours at 4ºC. Slides were washed in TBST for 5 minutes x 2 then placed in PBS. Cover slips were mounted with VectaMount mounting medium (Vector).

Slides were visualized for fluorescence using Olympus FV version 4.2 software on a Olympus confocal microscope 1000. 3 regions from each available slide were imaged. Images were saved as both .oib and .tif formats without any changes in image settings. Fluorescence was quantified on Image J. TIFF files for each brain region from the three cases were opened.

Total area fluorescence mean, area, and Raw Intensity were measures for all slides. Samples of background adjacent to each nucleus, as determined by FOX3 fluorescent signal, were measured. The average of all background samples were used to determine the mean background fluorescence. Adjusted total fluorescence of TRITC-pS129 αSyn for each image was determined

by subtracting the average background fluorescence from total mean fluorescence. A one-tailed t-test with Welch's correction for non-equal variance was used to compare the distribution and means between midbrain and thalamus of WNV positive cases and control cases.

### Quantification and statistical analysis

### Sample sizes

N represents the number of mice as described in legends of each figure.

### Statistical analysis

The Shapiro-Wilk test was first applied to test for normality. If normality was met, t-test or one-way analysis of variance (ANOVA) was used to prepare differences between two or more groups. If normality was not met, the non-parametric Mann-Whitney U test or Kruskal–Wallis one-way ANOVA was used to prepare differences between two or more groups. For parametric ANOVA tests, the Tukey's honestly significant difference (HSD) test was used for comparing differences between two groups post ANOVA with equal variances, and the Games-Howell post-hoc test used with unequal variances. For non-parametric ANOVA tests, the Dunn's test was used to compare between two group.

## Results

### Alpha-Synuclein supports immune gene expression in brain tissue

Following our previous work showing increased viral growth and increased mortality in αSyn KO mice upon viral infection[8], we analyzed the transcriptome of WT and αSyn KO whole mouse

345 brain tissue following infection with West Nile virus (WNV,1000 plaque forming units (pfu),
346 subcutaneous (sc) inoculation). Brains were harvested 8 days post infection with WNV or mock
347 infection, and RNA-seq was performed to determine differences in the transcription profile of
348 these treatment groups. The mock infected mice had highly similar profiles (**Supplementary**
349 **Fig. 1**), with only 4 genes having differential gene expression including the *Snca* gene (**Figure**
350 **1A**), indicating little variation in the gene expression of animals at basal state. Both genotypes of
351 mice exhibited marked alterations in their expression profiles following WNV infection. There
352 were 1317 upregulated genes in WT mice following WNV infection and 1785 upregulated genes
353 in αSyn KO mice following WNV infection (**Figure 1C,D**). In addition, there were 87
354 downregulated genes in the WNV-infected WT mice and 375 downregulated genes in the WNV-
355 infected αSyn KO mice (**Figure 1C,D**). Notably, we found substantially more upregulated and
356 downregulated genes in the KO mice compared to the WT mice suggesting greater dysregulation
357 of gene expression in the αSyn KO mice. When comparing WNV-infected, αSyn KO mouse
358 brain tissue to WT mice, we found 35 genes with significantly upregulated expression, and 50
359 genes that were significantly downregulated in the brain tissue of αSyn KO mice (**Figure 1B**).
360 Immune related genes that are important for antiviral responses were among the most
361 upregulated in both αSyn KO and WT mice. However, several immune-associated genes showed
362 significant downregulation in αSyn KO mice compared to WT mice including *Herc6, Tnfrsf25,*
363 and *Crip3* (**Supplemental Table 2**). These data together indicate that αSyn plays a role in gene
364 regulation during infection. We subsequently looked for immune genes that had lower
365 expression in WNV-infected, αSyn KO mice compared to WNV-infected, αSyn WT mice that
366 may explain a potential immune deficiency in the brain tissue that resulted in significantly
367 increased WNV growth in the brain as we have previously shown[8]. We found that *Herc6* (**Figure**

**1E**), *Oas1b* (**Figure 1F**), and *Ifit2* (**Figure 1G**) all showed evidence of decreased gene expression in the brain tissue of WNV-infected, αSyn KO mice compared to WNV-infected WT mice. Given the higher viral loads previously seen in the brain tissue of WNV-infected, αSyn KO mice compared to WNV-infected, WT mice,[8] we would expect to see greater expression of these genes in the αSyn KO mice if αSyn was not impacting gene expression. These data indicated that αSyn may have a role in regulation of innate immune gene expression in the brain following viral infection.

We next investigated the expression of ISGs in WNV-infected, αSyn KO and WT mice based on prior data showing that ISG expression in the brain is an important factor in WNV infection.[17-20] We infected αSyn KO and WT mice with WNV (1000pfu, sc) or mock infection, and we harvested whole brain at 8 days post infection. Using quantitative reverse transcription (qRT)-PCR analysis we evaluated the expression of 5 target ISGs that exhibited decreased expression in the RNAseq analysis of WNV-infected αSyn KO mice (*Oas1b, Irf9, Tlr3, Trim25,* and *Tgtp1*) and one gene that exhibited no significant changes in expression when comparing WNV-infected, αSyn KO and WT mice (*Mx1*). We found that WNV-infected, αSyn KO mice exhibited approximately a 2-fold decrease in expression of *Oas1b, Irf9, Tlr3,* and *Trim25* genes in brain tissue compared WT mice (**Figure 2A-D).** Similarly, WNV-infected, αSyn KO mice exhibited a 4-fold decrease in *Tgtp1* gene expression in brain tissue compared to WT mice (**Figure 2E).** Consistent with our transcriptomic analysis, we found that αSyn gene expression had no effect on *Mx1* gene expression in the brains of WNV-infected αSyn KO and WT mice (**Figure 2F**). These data supported our initial transcriptomic data showing that αSyn is required to support the full complement of ISG expression in brain tissue following WNV infection.

**Alpha-Synuclein Regulates Viral Growth Within Neurons**

We have previously shown that an attenuated alphavirus, Venezuelan equine encephalitis virus (VEEV) TC83, exhibits robust infection in the brains of αSyn KO mice following peripheral infection while causing no detectable infection in the brains of WT mice.[8] Prior studies have shown that VEEV TC83 strain is attenuated in part due to a mutation that reduces viral resistance to Ifit1 restriction, an interferon stimulated gene (ISG) that recognizes viral RNA and restricts translation[21]. To evaluate intrinsic αSyn-dependent inhibition of VEEV TC83 in brain tissue, we evaluated VEEV TC83 growth in the brain following intracranial inoculation in WT and αSyn KO mice. Over a four-day time course, we found a significant decrease in the weight of the αSyn KO mice (**Figure 3A**). At four days post-infection, we analyzed VEEV TC83 viral growth by plaque assay and found an 8.8 fold-increase (αSyn KO titer $2.27x10^6$ +/- $1.19x10^6$ pfu/mg, WT titer $2.48x10^5$ +/- $1.24x10^5$ pfu/mg, $p<0.05$) in viral growth in the brains of αSyn KO mice compared to WT mice (**Figure 3B**). These data indicate that VEEV-TC83 exhibits increased viral growth in brain tissue of αSyn KO mice independent of interactions with the blood brain barrier, and that αSyn functions within brain tissue separate from any peripheral immune response.

Since VEEV TC83 growth was restricted within the neuronal brain tissue, we next evaluated the basal expression of type 1 interferon receptor (IFNR) in brain tissue of αSyn KO to ensure that differences in viral growth and ISG expression were not due to decreased IFNR expression. Using brain tissue from mock-inoculated wild-type, αSyn KO, and IFNR KO mice, we used immune fluorescence on 3-4 mice per group to determine IFNR expression. We found that both wild-type and αSyn KO brain tissue exhibit high levels of IFNR compared to background signal found in IFNR KO mice (**Supplemental Fig. 2**).

Next, we determined whether neuron-specific expression of αSyn or microglia activation were contributing to the restriction of virus infection. Previous studies have shown that neuronal asyn expression can activate microglia and promote an inflammatory response.[22-24] Other studies indicate asyn-dependent activation of microglia occurs through a TLR-dependent mechanism. [23,25-29] Using both WNV and VEEV TC83 virus infections, we first determined the role of αSyn expression on the activation of microglia in the brain.

First, we evaluated the role of VEEV TC83 virus infection in activation of microglia in WT and KO mice. We have previously shown that VEEV TC83 infects the neurons of the CNS in αSyn KO mice but not in WT mice.[30] We injected WT and αSyn KO mice by intracerebral inoculation with VEEV TC83 (TC83, 1000pfu) and harvested brain tissue at 4 days post-infection, a time-point prior to significant peripheral inflammatory cell infiltration. Flow cytometry analysis of brain tissue revealed significantly increased numbers of CD45+CD11b+CD68+ cells, CD45+CD11b+IL6+ cells, and CD45+CD11b+TNFalpha+ following VEEV TC83 infection in both WT and asyn KO brain tissue, but we found no difference in CD45+CD11b+ cell activation when comparing WT and αSyn KO brain tissue (**Supplemental Fig. 3**). Next, we determined if microglial gene expression following VEEV TC83 infection in the brain was dependent on αSyn expression. Using the same TC83 infection experimental model as above, microglia were column isolated from brain tissue using CD11b antibody binding. Total RNA was extracted from CD11b+ brain cells and analyzed by PCR array for ISG expression (**Supplemental Fig. 3D**). ISG expression was normalized to mock-infected, WT microglia (CD11b+ cells) and revealed similar increases in ISGs following VEEV TC83 infection in both WT and αSyn KO mice. Based on these data, we αSyn-dependent regulation of

innate immune responses and ISG responses in the brain were not due to differences in microglia activation following virus infection.

Next, we utilized a human neuron culture model to evaluate the functional role of αSyn in ISG expression in human neurons.[13,31] WT and αSyn KO human embryonic stem cells (hESCs) were differentiated into human dopaminergic neurons as previously described.[13] Immunofluorescence microscopy for beta-III tubulin and tyrosine hydroxylase of differentiated WT and αSyn KO neurons indicated they have similar neuronal morphology and dopaminergic phenotype (**Figure 3C).** We infected αSyn KO and WT human dopaminergic neurons with VEEV TC83 virus at a MOI of 1 and determined viral titer in the supernatant at 0, 12, 24, 48, and 72 hours. We found a 2-fold increase in viral titer in the αSyn KO neurons after 24 hours (Figure 3D). By 72 hours, αSyn KO neurons exhibited a 19.9-fold increase in viral titer compared to WT neurons (αSyn KO titer $2.20x10^7$ +/- $1.49x10^7$ pfu/ml, WT titer $1.11x10^6$ +/- $1.05x10^6$ pfu/ml, $p<0.05$, **Figure 3D)**. These data show that αSyn expression inhibits viral growth in neurons and independent of other CNS cell types. These data and our gene expression data from αSyn KO mouse models suggested a cell autonomous role for αSyn in regulating interferon responses within neurons.

**Alpha-Synuclein modulates type I interferon responses in neurons**

To evaluate our hypothesis that αSyn expression supports innate immune responses within neurons, we treated αSyn KO and WT dopaminergic neurons with VEEV TC83 (MOI 10), poly I:C (25 μg/mL), and T1IFN (1000 IU/ml). We measured the expression level of *IFIT1* in all three conditions due to its importance in regulating VEEV TC83 infection, and the gene expression level of *Trim25* since it was decreased in WNV-infected αSyn KO mouse brain tissue compared to WNV-infected WT mice. We found that *IFIT1* expression was significantly

458 decreased by 4.8 fold in VEEV TC83-infected, αSyn KO neurons compared to WT neurons
459 ($p<0.0001$, ANOVA with Tukey's multiple comparisons, **Figure 4A**). In poly I:C-treated
460 dopaminergic neurons, we also found a significant 11.7-fold decrease in *IFIT1* expression in
461 αSyn KO neurons compared to WT neurons ($p=0.006$, ANOVA with Tukey's multiple
462 comparisons, **Figure 4B**). Following T1IFN treatment of dopaminergic neurons, we found a
463 smaller but significant 1.4-fold decrease in *IFIT1* expression in αSyn KO neurons compared to
464 WT neurons ($p<0.0001$, ANOVA with Tukey's multiple comparisons, **Figure 4C**).

465 Next, we evaluated *TRIM25* expression in αSyn KO and WT dopaminergic neurons and
466 found no significant changes in *TRIM25* gene expression following VEEV TC83 infection and
467 poly I:C treatment (**Figure 4D,E**). However, following treatment with T1IFN, we found that
468 αSyn KO neurons exhibited a 11.6-fold decrease in *TRIM25* expression compared to WT
469 neurons ($p=0.005$, ANOVA with Tukey's multiple comparisons, **Figure 4F**). These data suggest
470 that αSyn-dependent modulation of ISG responses may be occurring at the level of T1IFN
471 signaling since *IFIT1* and *TRIM25* expression were decreased in αSyn KO neurons following
472 T1IFN treatment.

473 To evaluate the role of αSyn in T1IFN signaling, we treated αSyn KO and WT human
474 dopaminergic neurons with type I IFN (10,000 IU/ml) and collected RNA from cells at 4 hours
475 post-treatment. ISG expression analysis was performed using a $RT^2$ Profiler PCR array that
476 measures the expression level of 91 genes associated with the T1IFN signaling. We compared
477 the expression level of these genes between IFN-treated WT and αSyn KO cells, using a mock
478 treatment of WT cells as a control. We found that expression of most ISGs in the panel were
479 significantly decreased in the IFN-treated αSyn KO neurons compared to WT neurons (**Figure**
480 **5A**). In total, 61 of the assayed genes had significantly lower expression levels in αSyn KO

neurons, including important mediators of the type I IFN pathway such as *STAT1* and *IRF9*, and important innate immune genes including *IFIT1*, *TLR3*, and *IFNAR2* (**Figure 5B**). Of note, several genes critical to the innate immune response in the brain (*IFNAR1, IRF9, JAK1, IL6,* and *OAS2*) were among the most sensitive to loss of αSyn expression with over 100-fold lower gene expression in αSyn KO neurons (**Supplemental Table 3**). These data indicate that αSyn expression is critically important for T1IFN-dependent gene expression in neurons.

**Alpha-Synuclein supports type 1 interferon signaling in neurons**

Having established that αSyn supports T1IFN-dependent gene expression, we sought to define the mechanisms linking αSyn to interferon signaling. We first determined whether interferon induced changes in αSyn localization within human neurons. RC17 human embryonic stem cells (hESCs) were differentiated into cortical neurons as described above and verified to exhibit cortical neuronal markers using immunostaining for Tbr1 and Ctip2 at day 55(**Supplemental Fig. 4**). Cortical neurons were treated with type 1 interferon A2 (IFNa2, 10,000IU/mL) or with a media-only control for 4 hours. Cortical neurons were fixed and immunostained using primary antibodies to total αSyn and beta-III tubulin. We found that cortical neurons treated with IFNa2 exhibited significantly increased nuclear expression of αSyn compared to control treated cortical neurons (**Figure 6**, N=8 per group, unpaired t-test, **p=0.0085, ****p<0.001).

Since interferon treatment induced nuclear localization of αSyn, we next determined the interactions between αSyn and type 1 interferon signaling proteins over a time course. STAT2 is phosphorylated upon T1IFN stimulation at the interferon receptor resulting in formation of a heterotrimer with phospho-STAT1 (P-STAT1) and IRF9.[32] Due to the large number of neurons needed for these studies, we utilized primary murine cortical neurons as previously described.[16]

Primary murine cortical neurons were treated with mock control (PBS) or interferon a2 and harvested for immune fluorescence analysis at 1 and 4 hours post-treatment. At both 1 and 4 hours after interferon a2 treatment, αSyn in cortical neurons exhibited significantly increased co-localized signal with phosphorylated-Stat2(Tyr690) (**Figure 7A-D**, N=3 experiments and 12-17 images per group, ANOVA of Manders2 signal co-localization with Costes P=1, *p<0.0001, Tukey's multiple comparisons test).

To further investigate αSyn and STAT2 interactions in human dopaminergic neurons, WT and αSyn KO human dopaminergic neurons were plated and differentiated for 42 days. We treated the neurons with T1IFN a2 (10,000 IU/ml) or phosphate buffered saline (PBS) vehicle control for 4 hours. Similar to our findings in human cortical neurons, we found αSyn nuclear localization in T1IFN-treated human neurons (**Supplemental Fig. 5A**). To quantify this observation, the images were randomized and analyzed for nuclear localization by an individual blinded to the treatment groups using mean fluorescence intensity (MFI) of αSyn signal in the nucleus. T1IFN-treated human dopaminergic neurons exhibited a significant increase in αSyn MFI in the nucleus compared to vehicle-treated neurons (**Supplemental Fig. 5B**, unpaired t-test, N=6-8 per group in 2 experimental replicates, *p=0.0225). Using human dopaminergic neurons, we next determined whether phosphorylation of STAT2 was dependent on αSyn expression. WT and αSyn KO dopaminergic human neurons were treated with control or interferon a2 as above and harvested at 4 hours post-treatment for phospho-STAT2 and total STAT2 ELISA. We found that human dopaminergic αSyn KO neurons exhibit significantly decreased phopho-STAT2 expression compared to WT neurons with no effect on total STAT2 expression (**Figure 7E-F**, N=5 per group in 2 experimental replicates, ANOVA, *p=0.0251).

Since αSyn exhibited evidence of co-localization and regulation of pSTAT2 expression, we next evaluated αSyn co-localization with phosphorylated Stat1 (pStat1) and the type 1 interferon receptor (IFNR) in murine cortical neurons as described above. Primary murine cortical neurons were treated with mock control (PBS) or interferon a2 and harvested for immune fluorescence analysis at 1 and 4 hours post-treatment. We found that αSyn co-localized with pStat1(Tyr701) at 1 hour post-treatment but not at 4hours post-treatment (**Supplemental Fig. 6**, N=15-20 per group in 3 experimental replicates, one-way ANOVA with multiple comparisons test of Manders2 values with Costes P=1, *p<0.0001). Subsequent analysis of αSyn co-localization with the type 1 interferon receptor (IFNR) in interferon treated mouse cortical neurons revealed no evidence of co-localization between the two signals (**Supplemental Fig. 7**).

**Viral Infection Induces Phosphorylation of Alpha-Synuclein**

We next investigated whether phosphorylation at the S129 position was associated with viral infection in human brain tissue. Following acute viral infection in the brain, T1IFN responses are an important primary innate immune response and critical to limiting virus-induced cell death.[33,34] Phospho-S129 αSyn is commonly found in Lewy bodies in Parkinson's disease and is a hallmark characteristic of the condition,[35] but the expression pattern and levels of pS129 αSyn is not known in human cases of viral encephalitis. We obtained sections from subcortical brain tissue from 5 patients with a pathological and clinical diagnosis of WNV encephalitis and 4 control patients and evaluated expression of total and pS129 αSyn in human brain tissue (**Supplemental Table 4).** We completed immunofluorescence imaging analysis for pS129 αSyn expression in subcortical grey matter from control patients (**Figure 8A**), subcortical grey matter

548 from WNV infected (**Figure 8B)** patients, and white matter from the same WNV-infected
549 patients (**Figure 8C**). Since WNV infects subcortical grey matter and not white matter, the
550 histology staining of white matter provided an internal control for virus-associated αSyn
551 expression. Using grader-blinded scoring for MFI in each patient, we found that phospho-S129
552 αSyn expression is significantly increased in WNV-infected subcortical grey matter compared to
553 control brain tissue and brain tissue from white matter regions (**Figure 8D,** control tissue N= 3,
554 WNV+ grey matter tissue N=6, white matter tissue N=3, $p < 0.05$). These data show for the first
555 time in humans that viral infection leads to phosphorylation of αSyn at Serine 129, a post-
556 translational modification of αSyn associated with Parkinson's disease.

557 **Discussion**

558 αSyn is a highly conserved protein expressed in neurons of most vertebrate species. Its
559 unique localization to nuclei as well as synaptic puncta suggests it may have functions beyond
560 synaptic biology. Mounting evidence supports an important role for αSyn in protection of
561 neurons but the mechanisms of protection remain unclear.[8,11,12] Our data show that during virus
562 infection, αSyn supports expression of ISGs required to inhibit viral infection. This was
563 confirmed both *in vivo* in murine infection models and in a human neuronal culture system.
564 Moreover, the same ISGs stimulated by virus infection are also dependent on αSyn for
565 expression following poly I:C and type I interferon treatment. The importance of αSyn-
566 dependent ISG expression is supported by our findings showing that VEEV TC83 growth is
567 significantly increased in αSyn KO neurons and in αSyn KO brain tissue compared to WT
568 neurons and mice, respectively. VEEV TC83 is attenuated due to a mutation in a 5' untranslated
569 region that results in increased IFIT1 restriction of viral growth[36], and our discovery of increased
570 viral growth in the absence of αSyn expression suggested that IFIT1 activity was decreased in

αSyn KO neurons. We subsequently found evidence of decreased *IFIT1* expression in αSyn KO neurons following VEEV TC83 infection and following treatment with poly I:C. These data show that αSyn expression supports ISG responses that are critical to control RNA virus infection in neurons.

We also show that VEEV TC83 infection and poly I:C treatment did not significantly increase expression of *TRIM25* in WT human neurons, while treatment with T1IFN resulted in significantly increased expression of *TRIM25*. In human αSyn KO neurons, expression of *TRIM25* was significantly decreased compared to WT neurons following treatment with T1IFN. Since αSyn-dependent expression of *TRIM25* occurs down stream of T1IFN stimulation but not poly I:C stimulation, we hypothesized that αSyn interacts with T1IFN signaling pathways to support and regulate ISG expression in neurons. We investigated this by measuring the expression of several key components of the interferon and STAT signaling pathways following T1IFN treatment of neurons. Using a PCR array for ISG expression, we found a broad decrease in ISG expression in αSyn KO human neurons following T1IFN stimulation. This finding provides insight into the mechanism by which mice and neurons lacking αSyn expression are deficient in combating viral growth since T1IFN-dependent expression of ISGs are a primary mechanism immune restriction in the central nervous system.[37]

We next investigated the mechanism by which αSyn expression alters ISG expression. Following type I IFN treatment of αSyn KO and αSyn WT human neurons, our data show that αSyn co-localizes with phosphorylated Stat2, and we found reduced levels of P-STAT2 protein when αSyn expression was knocked out of human neurons. These data suggest that αSyn localizes with P-STAT2 following interferon stimulation and supports activation. Interestingly, we also found that P-STAT1 co-localized with αSyn at 1 hour post-treatment, and that αSyn localizes to the

nucleus following interferon treatment. Previous studies have reported the nuclear translocation of αSyn under conditions of oxidative stress[38] and exposure to toxins, such as paraquat.[39] However, αSyn nuclear localization has not been described in response to a signal transduction pathway, such as the IFN pathway. This is the first report that αSyn is translocating to the nucleus in association with STAT activation to support ISG expression in neurons. To confirm this observation in human tissue, we investigated the localization and phosphorylation status (S129) of αSyn in virus-infected human brain tissue. We found that pS129 αSyn levels increase in human neural tissue following WNV infection.

These data confirmed our findings that αSyn responds to infection and inflammatory pathways by localizing to the nucleus and suggest that pS129 αSyn has a functional role in the neuronal nucleus following T1IFN stimulation. The specific interactions of pS129 αSyn in the nucleus are not known but are an important future direction for this work. Studies have suggested that αSyn has a role in histone modification to facilitate transcription and it may regulate NF-kB expression in models with overexpression αSyn.[40,41] Thus, we hypothesize that, during type 1 interferon stimulation in neurons, αSyn is phosphorylated, interacts directly with pSTAT2 to support nuclear translocation of the pSTAT1-pSTAT2 heterodimer, and thereby directly facilitates transcription of ISGs. This role of αSyn in supporting T1IFN responses seems to be specific to fully differentiated neurons as we have been unable to replicate these phenotypes in non-neuron cell types.

These data also provide a mechanism for our previously reported observations that mice deficient in αSyn fail to control neuroinvasive viral infection, and these data give a new insight into a common pathway for potential triggers of αSyn expression over time that may be involved in the pathogenesis of PD and other synucleinopathies. Our data also indicate that T1IFN

responses increase expression of phosphorylated S129 αSyn. Future studies will need to determine the role of interferon stimulation formation of PD-related fibrils that also contain pS129 αSyn. Further studies evaluating the interactions between interferon signaling and αSyn post-translational modifications are needed to define the role of αSyn-dependent T1IFN signaling events in the pathogenesis of synucleinopathies like PD.


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**Competing Interests**

The authors report no competing interests.

Supplementary Material is available at *Brain* online.

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## Figure Legends

**Figure. 1. RNAseq analysis reveals a group of virus-induced genes that are dependent on alpha-synuclein expression in brain**. C57BL6/J mice were treated with mock or WNV (1000pfu/sc) inoculum and brain tissue harvested at day 8 post-infection for RNAseq analysis of whole brain tissue. Volcano plots showing comparative gene expression following infection were created comparing **A)** Mock infected KO compared to mock infected WT mice, **B)** WNV infected KO compared to WNV infected WT mice, **C)** WNV infected WT compared to mock infected WT mice, and **D)** WNV infected KO compared to mock infected KO mice. Mean FPKM values for **E)** *Herc6,* **F**) *Oas1b*, and **G)** *Ifit2* are reduced in WNV-infected KO brain tissue compared to WT brain tissue. N=4 mice/group. WT= wild-type mice, KO=αSyn knockout mice. *$p<0.01$. ANOVA with Tukey's multiple comparisons.

**Figure 2. Alpha-synuclein knockout mice exhibit decreased expression of select interferon-stimulated genes in the brain following WNV infection**. QPCR values from brain tissue is shown in indicated treatment groups to quantify expression of ISGs identified in RNAseq analysis including: A) Oas1b, B) Irf9, C) Tlr3, D) Trim25, and E) Tgtp1. F) QPCR values for Mx1 in brain tissue of mice as an example of an ISG in the brain not altered by αSyn expression.

N=3 mice per treatment group. WT= wild-type mice, KO=αSyn knockout mice. *p<0.005, two-way ANOVA

**Figure 3. Alpha-synuclein expression restricts VEEV TC83 infection in murine brain tissue and human dopaminergic neurons.** Wild-type (WT) and αSyn knockout (KO) mice were inoculated with VEEV TC83 ($10^5$ pfu, intracerebral inoculation) and **A)** followed for weight loss over the indicated time period. N=6 mice per group. *p<0.0001, Two-way ANOVA. **B)** At 4 days post-infection with VEEV TC83, brain tissue was analyzed for VEEV TC83 viral titer using plaque assay. N=4-6 mice per group. *p=0.038. Mann-Whitney U test. **C**) WT and αSyn KO human dopaminergic neurons, differentiated for 42 days, were positive for beta-III tubulin (red) and tyrosine hydroxylase (green). **D**) WT and αSyn KO neurons were inoculated with VEEV TC83 (MOI 1) and supernatant collected at indicated time points for viral titer assay. *p<0.05, two-way ANOVA. N=4 replicates per group.

**Figure 4. Alpha-synuclein expression supports IFIT1 and TRIM25 gene expression in human neurons**. WT and αSyn KO neurons were inoculated with mock or VEEV TC83 (MOI 10) and neurons were harvested at 12 hours post-infection for gene expression analysis. **A**) Following VEEV TC83 infection, *IFIT1* gene expression increased 17-fold in WT neurons and 6-fold in αSyn KO neurons. ** p<0.01 **B**) Poly I:C induced *IFIT1* expression 100-fold in WT neurons and 3-fold in αSyn KO neurons. **p<0.01 **C**) *IFIT1* gene expression increased 250-fold with T1IFN treatment in WT neurons and 62-fold in αSyn KO neurons. N=3-5 per group. *p<0.05, ANOVA with Tukey's multiple comparisons. **D)** *TRIM25* gene expression was not increased following VEEV TC83 infection. N=4-5 replicates per group. Next, WT and αSyn KO

780 neurons were treated with poly I:C (25 microg/ml) and neurons harvested at 4 hours post-
781 treatment for QPCR analysis. **E)** *TRIM25* gene expression was not significantly increased by
782 Poly I:C treatment. N=3-4 per group. WT and αSyn KO neurons were treated with type 1
783 interferon (T1IFN, 1000IU/ml) and cells harvested at 4 hours post-treatment for QPCR
784 analysis. **F**) *TRIM25* gene expression increased 32-fold in WT neurons with T1IFN treatment
785 and 1.9-fold in αSyn KO neurons. **p<0.05, ANOVA, Tukey's multiple comparisons. N=3-5
786 per group.
787
788 **Figure 5. Alpha-synuclein expression supports type 1 interferon-dependent interferon**
789 **stimulated gene expression in human dopaminergic neurons.** WT and αSyn KO neurons
790 were treated with type 1 interferon (T1IFN, 10000IU/ml) and cells harvested at 4 hours post-
791 treatment for analysis using ISG expression PCR array. **A**) Heat map of ISGs showing relative
792 gene expression compared to control-treated WT neurons. Blue=decreased expression, black=no
793 change, and red = increased expression. **B**) Graphic representation of ISG relative gene
794 expression comparing αSyn KO neurons (y-axis) to αSyn WT neurons (x-axis).
795 **Figure 6. Alpha-synuclein localizes to the nucleus following type 1 interferon stimulation.**
796 Human cortical neurons were treated with **A)** media control or **B)** type 1 interferon (IFNa2,
797 10,000 IU/ml). At 4 hours post-treatment, neurons were analyzed for expression total alpha-
798 synuclein (asyn, green), a neuron marker (TUJ1, red), and dapi. **C)** The percent of cortical
799 neurons expressing asyn in the nucleus per image was determined (**p=0.0085, N=8 ). **D)** Mean
800 fluorescence intensity of asyn in the nuclease was also measured (****p<0.0001, N=8).
801

**Figure 7. Alpha-synuclein localizes with pSTAT2 and supports PSTAT2 expression.** Mouse cortical neurons were treated with **A)** control or type 1 interferon (IFNa2, 10,000 IU/mL) for **B)** 1 hour or **C)** 2 hours and labeled using immunoflouresence analysis for asyn (red) and pSTAT2 (green) expression. **D)** Cortical neurons were analyzed for asyn co-localization with pSTAT2 using FIJI Co-loc analysis for Manders2 ratios of asyn signal that co-localizes with pSTAT2 signal (*$p<0.0001$, N=12-17 per group). **E-F)** Human dopaminergic neurons expression asyn (WT) or with CRISPR-mediated knockout of asyn (asyn KO) were treated with IFNa2 (10,000 IU/mL) or control and harvested at 4 hours post-treatment for ELISA analysis of pSTAT2 and total STAT2 expression (WT + control N = 4, WT + T1IFN N = 5, ASyn KO + T1IFN N = 5, $p<$ 0.05 two-sided ANOVA).

**Figure 8. Viral infection of human brain tissue results in increased expression of phospho-S129 alpha-synuclein.** Immunofluorescent histology for phospho-S129 asyn (red) of **A**) control human brain tissue (bar=50μM), **B**) WNV-infected human thalamus(bar=200μM), and **C**) frontal white matter (bar=200μM). Blue=DAPI nuclear staining. **E**) Measure of fluorescence intensity units per field of phopho-S129 asyn staining of human brain tissue acutely infected with West Nile virus (WNV). Each data point represents an individual patient sample in the indicated brain region. *$p<0.05$ ANOVA.