Supplementary Information

Differential innate immune response programs in neuronal subtypes determine susceptibility to infection in the brain by positive stranded RNA viruses

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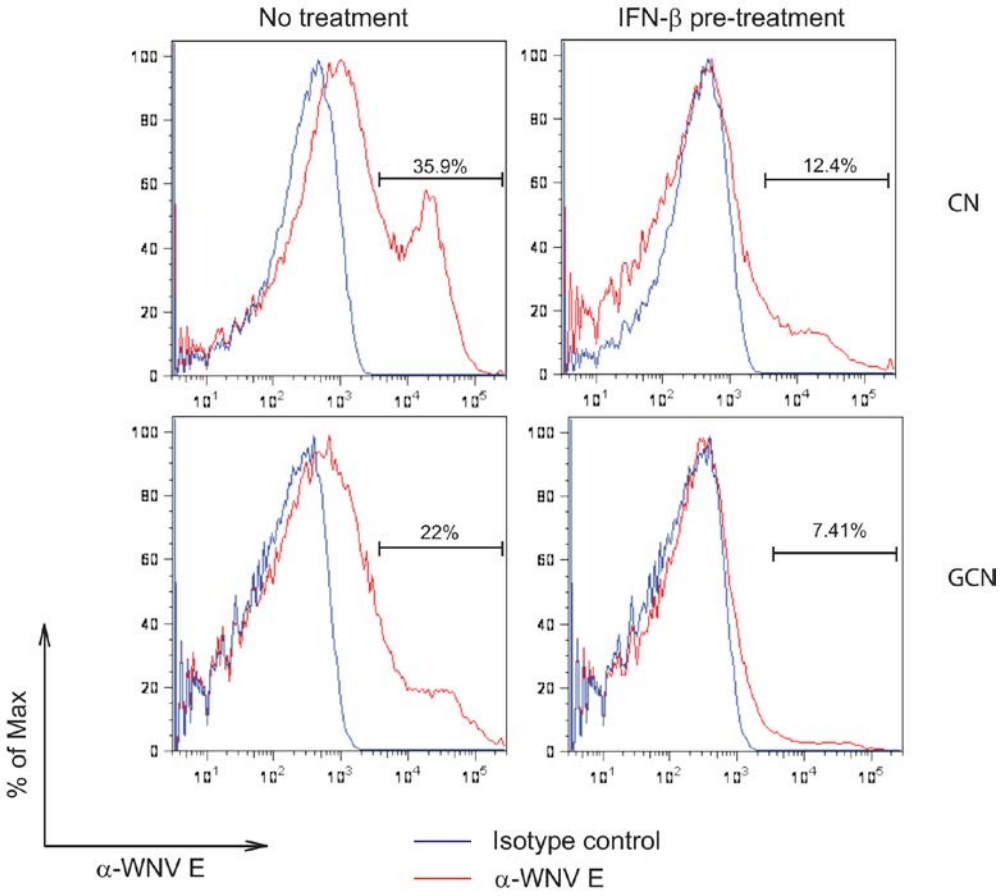
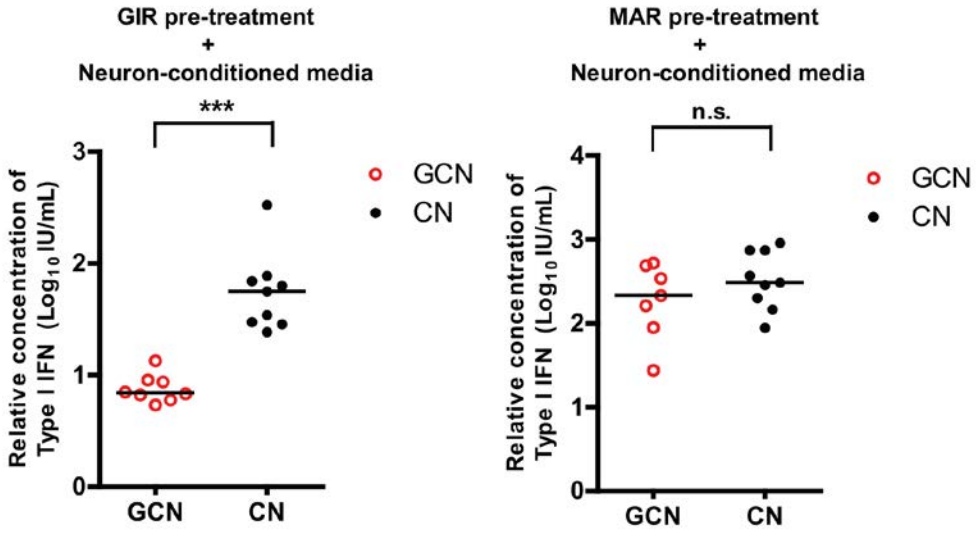
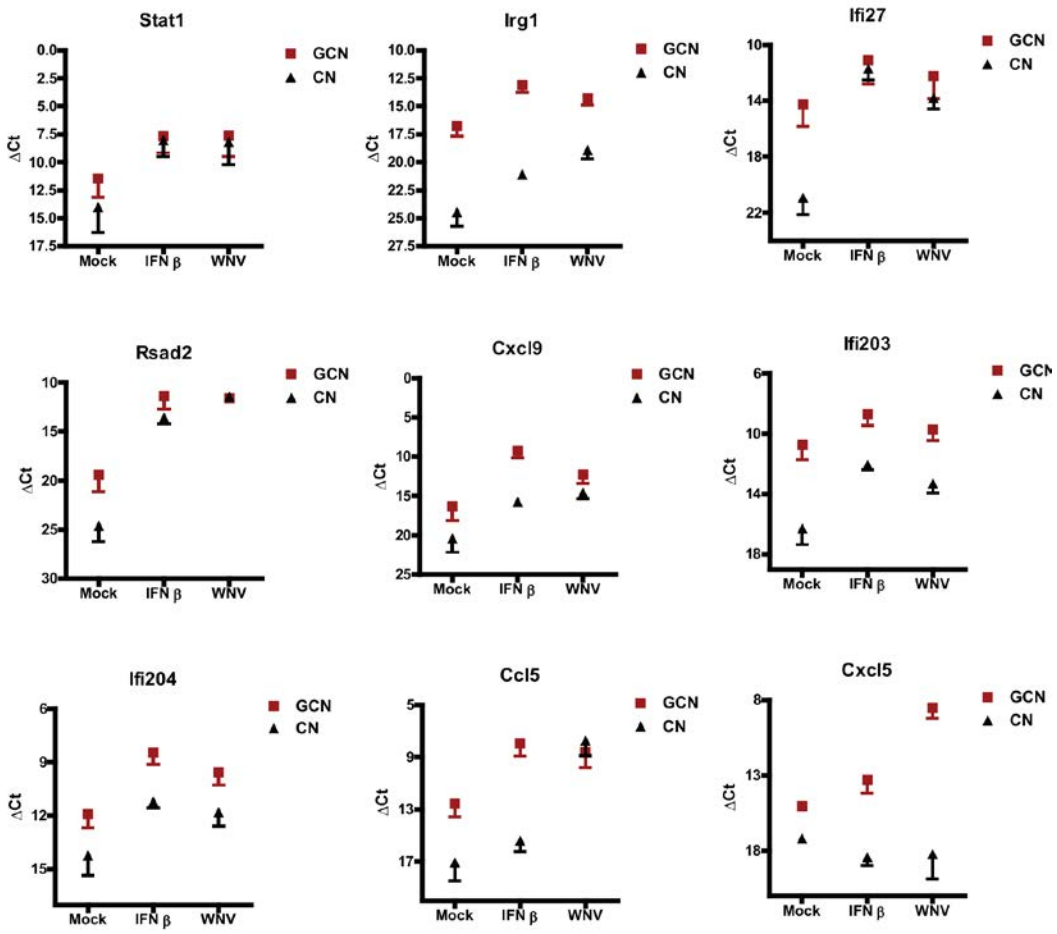


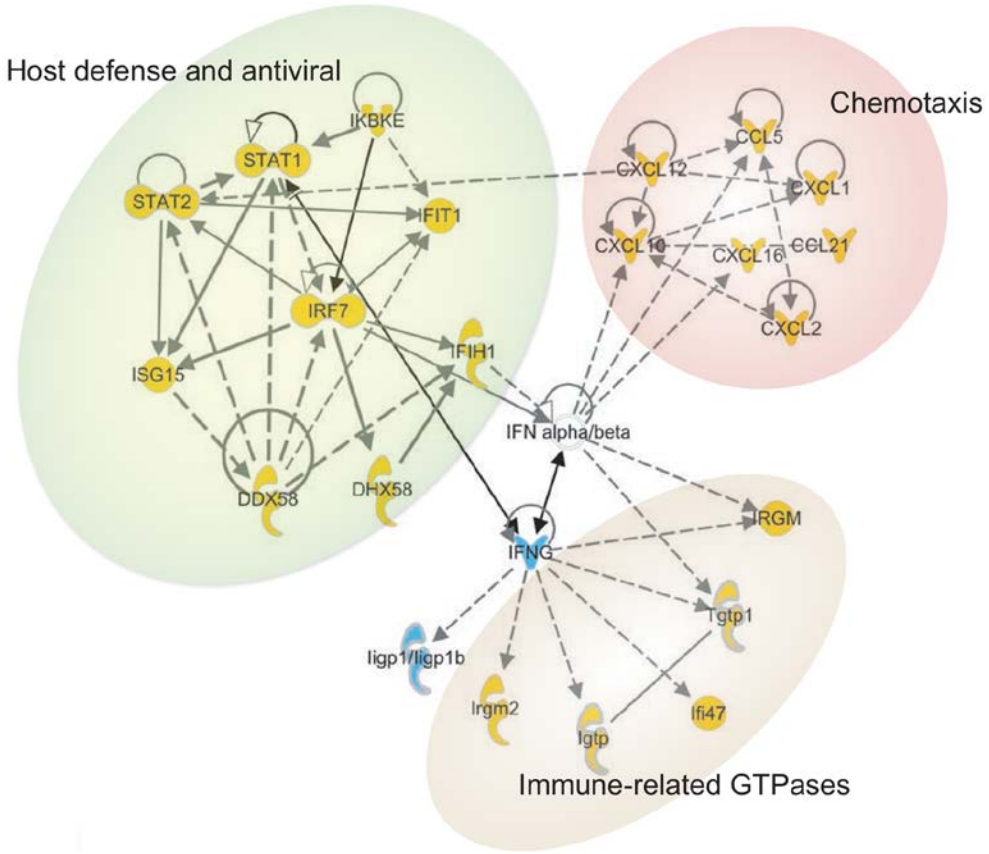
Supplementary Figure 1. Flow cytometry analysis on infected GCN and CN. Flow cytometry analysis of GCN and CN infected with WNV (MOI of 0.1) for 24 hours with or without IFN-β pre-treatment (10 IU/mL, 12 hours). The results are representative of two independent experiments.



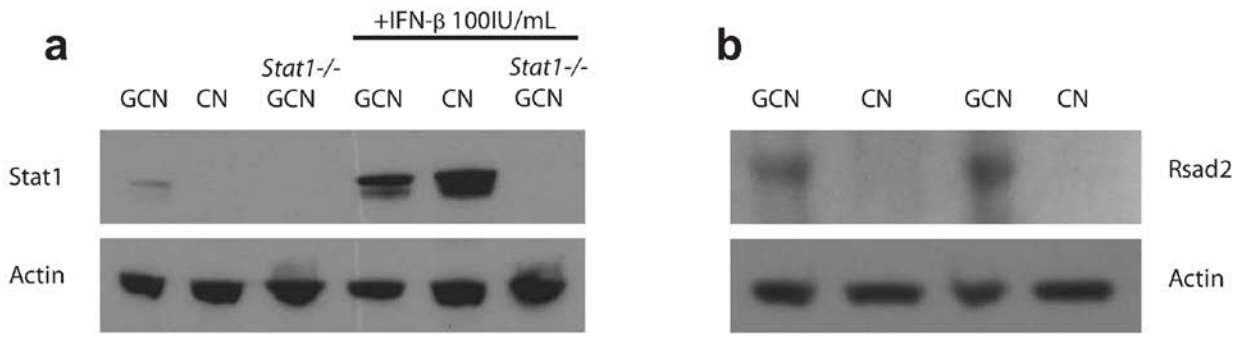
Supplementary Figure 2. Relative concentration of type I IFN in the supernatants of cultured GCN and CN. GCN and CN were infected with WNV (MOI of 0.1) and type I IFN level in the supernatants was determined by an encephalomyocarditis virus cytopathic effect bioassay on L929 cells[1]. To confirm type I IFN-mediated effect, L929 cells were pre-treated with 25 μg/ml of MAR1-5A3 (MAR) anti-Ifnar MAb or an isotype control MAb (GIR-208 (GIR)). Results are representative of two independent experiments and asterisks (***, $P < 0.0001$) indicate differences that are statistically significant by the Mann-Whitney test.



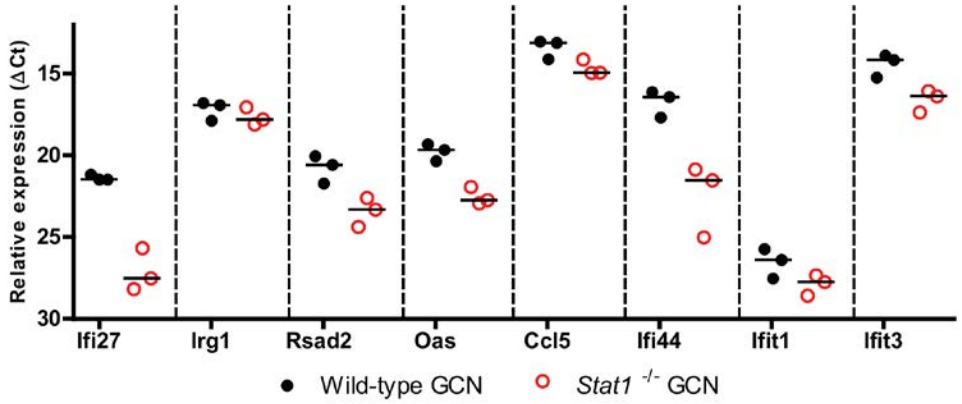
Supplementary Figure 3. Validation of differentially expressed ISGs by qRT-PCR. qRT-PCR validation of differential gene expression patterns in GCN and CN. Shown are nine selected genes that showed distinct expression patterns at basal level and after IFN-β treatment or WNV infection in GCN and CN. The results are representative of at least two independent experiments performed at least in duplicate. The data is displayed as the ΔCt value compared to cellular levels of 18S ribosomal RNA. Primer sequences are listed in **Supplementary Table 3.**



Supplementary Figure 4. Canonical PRR and IFN signaling pathway genes are expressed in GCN at the basal level. Genes in canonical PRR and IFN signaling pathways that are highly expressed in GCN at the basal level were determined by Ingenuity Pathway Analysis. Yellow color indicates higher basal expression level in GCN compared to CN within cutoff values of a ≥2-fold change and an ANOVA *P* value with a Benjamini and Hochberg algorithm-corrected false discovery rate of 0.01. Fold differences in expression values between GCN and CN are listed in **Supplementary Table 2.** Unique molecular signatures associated with host defense responses (e.g., immune-related GTPases) in GCN also were determined by Ingenuity Pathway Analysis. Shown are functional relationships that associate with antiviral activity, immune-related GTPases, and chemotaxis. Genes colored in blue show lower basal expression levels in GCN compared to CN.

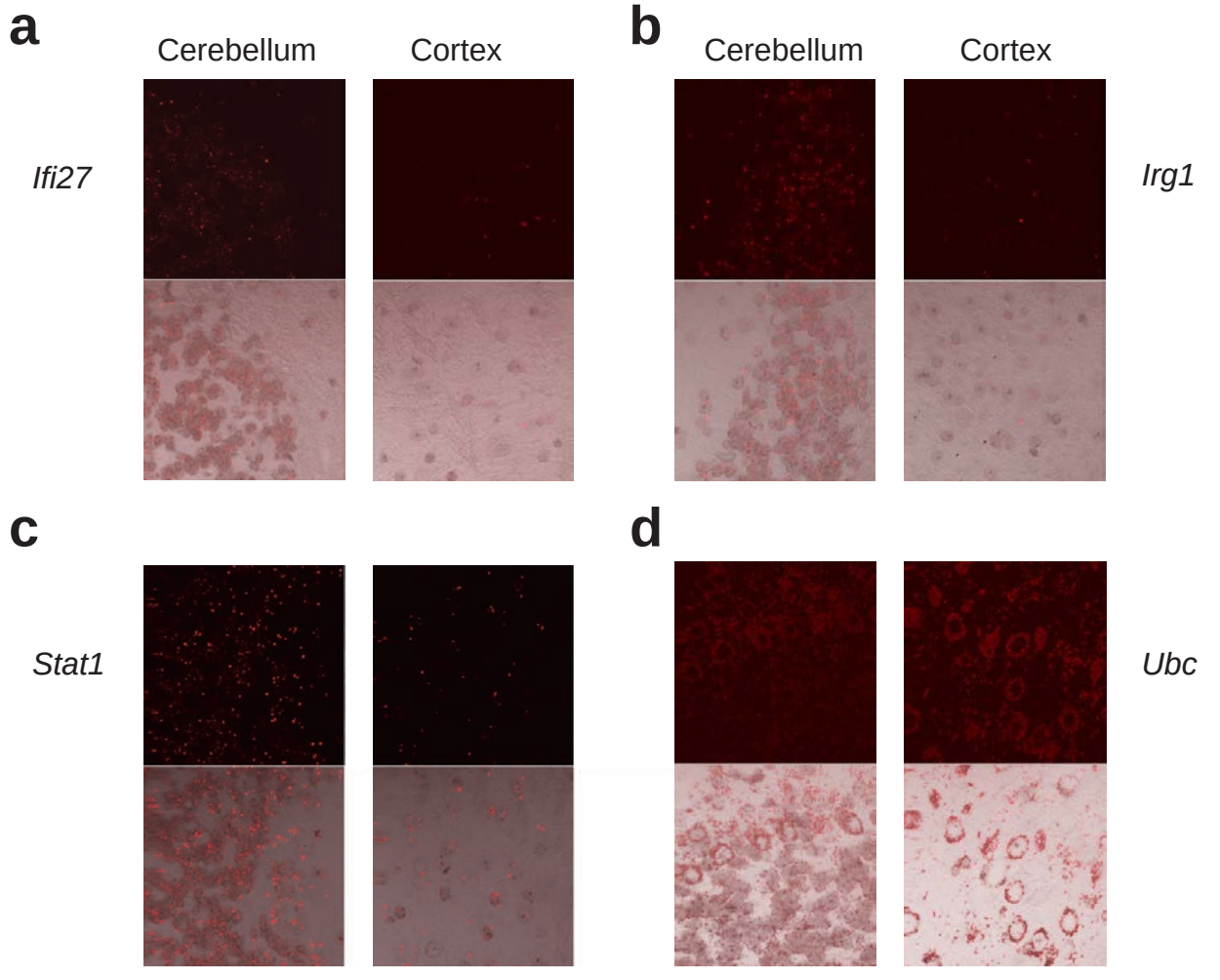


Supplementary Figure 5. Stat1 and Rsad2 are expressed differentially at the protein level in GCN and CN. Cells were left untreated or treated with 100 IU/mL of IFN-β for 24 hours, and lysates were harvested in Laemmli sample buffer supplemented with 5% of β-mercaptoethanol and separated by 10% SDS-PAGE. Immunoblot analysis was performed using antibodies against Stat1, β-actin, and Rsad2. *Stat1*$^{-/-}$ GCN were used as a negative control for blotting of Stat1 protein. The results are representative of two independent experiments.

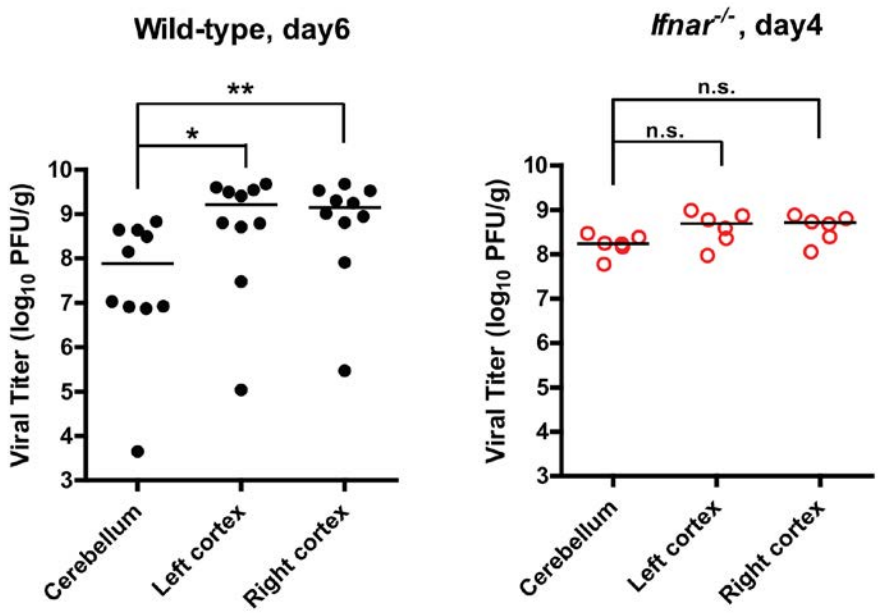


Supplementary Figure 6. ISGs expression level is lower in *Stat1*$^{-/-}$ GCN compared to wild-type GCN.

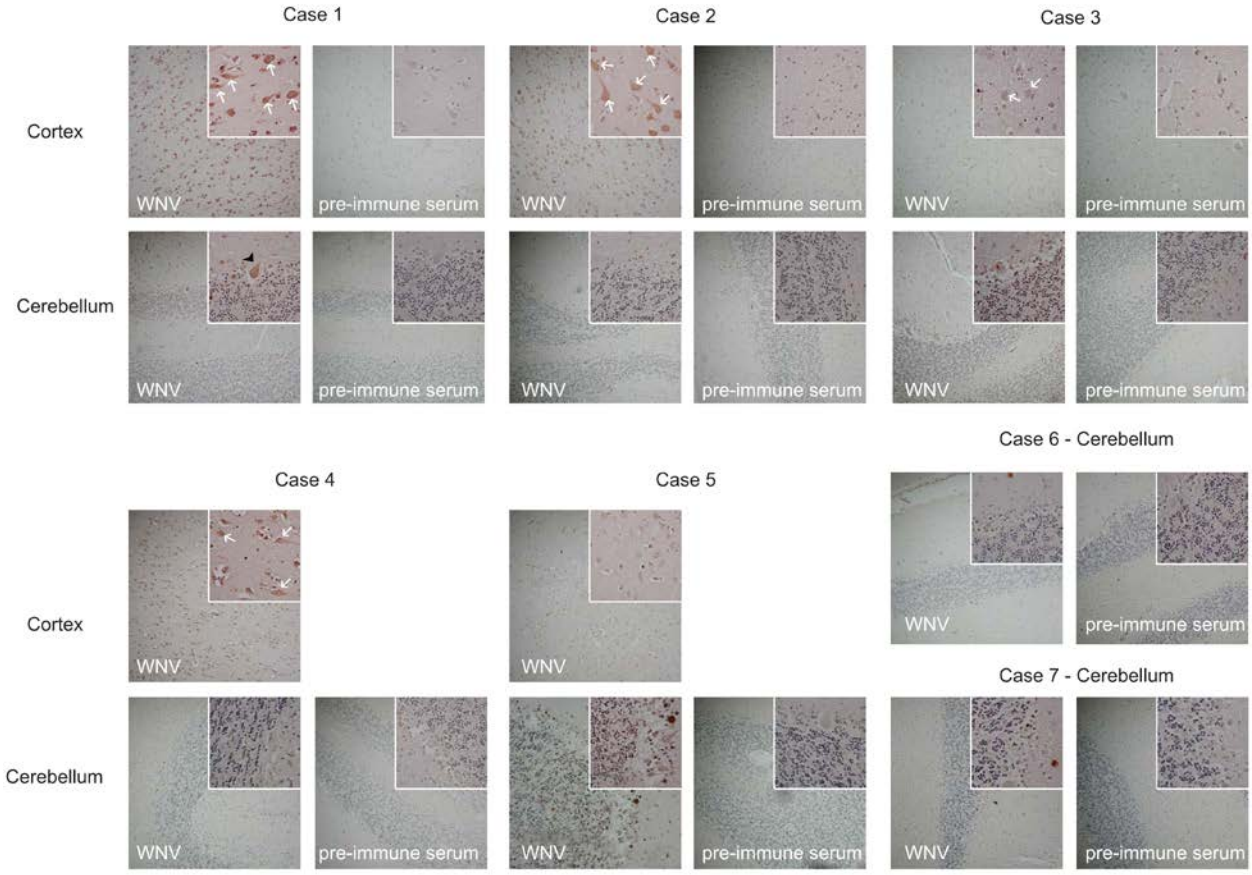
RNA was harvested from *Stat1*$^{-/-}$ and wild-type GCN, and ISG expression levels were compared by qRT-PCR. The data is displayed as the ΔCt value compared to cellular levels of 18S ribosomal RNA.



Supplementary Figure 7. Differential ISG expression in distinct regions of the brain in naïve mice. Differential gene expression of (**a**) *Ifi27*, (**b**) *Irg1*, and (**c**) *Stat1* in the cerebellum and cortex of naïve wild-type mice was determined by fluorescence *in situ* RNA hybridization. (**d**) *Ubc*, a housekeeping gene involved in cellular ubiqutination, was used as a positive control of staining. *Dapb*, a gene from E. coli, was used as a negative control (data not shown).



Supplementary Figure 8. WNV infection of different neurons in the cerebral cortex and cerebellum from brains of wild-type and *Ifnar*$^{-/-}$ mice. Brains from wild type and *Ifnar*$^{-/-}$ C57BL/6 mice were harvested at day 6 (wild type) or day 4 (*Ifnar*$^{-/-}$) after i.c. infection with 10^1 PFU of WNV. Viral titer was measured by plaque-forming assay on BHK21-15 cells. Solid lines represent the median viral titers and reflect data from six to ten mice per genotype.



Supplementary Figure 9. Infection of neurons in the cerebral cortex and cerebellum from brains of fatal human WNV cases. Brain sections from seven fatal human cases of WNV encephalitis were stained with rat anti-WNV antisera (*brown*) or pre-immune sera. Infected CN and Purkinje neurons of the cerebellum are indicated by white arrows and black arrow heads, respectively. Insets are high-power images of each case. Due to the limited supply of autopsy samples, images from some sections were not available.

Supplementary Table 1. Top four biological pathways associated with genes induced by IFN-β treatment in both GCN and CN

Pathway annotation	Genes	*P* value
1. Interferon signaling	*Ifit3, Socs1, Oas1, Mx1, Ifi35, Stat2, Irf9, Psmb8, Stat1, Tap1, Irf1*	1.41E-14
2. Antigen presentation pathway	*B2m,Psmb9,HLA-A,HLA-E,HLA-B,Psmb8,HLA-G,Tap1,Tap2,Tapbp, HLA-C*	4.10E-14
3. Pattern recognition receptors in recognition of bacteria and viruses	*Oas1d,Oas1,Oas2,Oas1b,Ccl5,Oas1f,Oas3,Ifih1,Irf7,Ddx58,Casp1,Nod1,Eif2ak2,Tlr3*	5.39E-13
4. Activation of IRF by cytosolic pattern recognition receptors	*Dhx58,Ifih1,Irf7,Ddx58,Zbp1,Stat2,Irf9, Stat1,Adar,Ifit2,Isg15*	2.37E-11

Biological functions were determined by Ingenuity® Systems Pathway Analysis of the 246 genes that were induced in both GCN and CN after IFN-β treatment. Right-tailed Fisher's exact test was used to calculate a *P* value.

Supplementary Table 2. Fold differences in expression of key innate defense genes between GCN and CN

(a) Candidate innate defense genes selected based on literature and Ingenuity® Systems Pathway Analysis

	Gene name	Fold difference of basal level (GCN vs. CN)	Fold change after IFN-β treatment	
			GCN	CN
*	Bst2/Tetherin	6.63	88.8	167
	Bcl2l14	5.85	< 2	< 2
	Casp1	3.31	2.65	3.84
*	Casp12	7.68	< 2	3.16
	Ccl21	30.8	2.54	< 2
	Ccl4	19.3	< 2	< 2
	Ccl5	24.8	6.26	4.67
	Ccl7	61.0	4.29	9.17
	Cxcl1	14.0	< 2	2.90
	Cxcl10	3.52	37.1	79.1
	Cxcl12	3.35	< 2	< 2
	Cxcl16	31.9	2.16	3.39
	Cxcl2	14.0	< 2	< 2
*	Lgp2	2.90	22.4	14.5
	Hpse	16.6	< 2	< 2
*	Ifi203	12.4	8.30	15.1
*	Ifi27	58.1	7.82	133
*	Ifi35	3.04	32.8	53.1
*	Ifi44	10.3	19.2	69.6
*	Ifi47	4.25	75.8	362
*	Ifit1	25.5	455	2880
*	Ifit3	5.63	167	924
*	Ifitm3	3.20	21.8	42.2
	Ifna2	2.54	< 2	3.57
	Ifng	-2.43	< 2	< 2
*	Igtp	9.38	56.8	104
*	Iigp1/Iigp1b	-2.35	239	250

	Ikbke	4.59		
	Ikbke	4.59	< 2	< 2
	Il1b	6.06	< 2	< 2
	Irf7	4.71	189	252
	Irf9	2.78	8.77	15.4
*	Irgm2	10.0	26.8	59.9
*	Isg15	6.44	87.9	500
	Jak1	2.05	< 2	< 2
	Ifih1(Mda5)	2.78	32.2	75.8
	Mx1	5.85	60.6	80.2
	Nod1	2.37	3.79	2.66
	Nod2	2.40	< 2	< 2
	Nfkb1	2.67	< 2	< 2
	Oas1	12.5	55.5	321
	P2ry6	9.63	< 2	< 2
	Phf15	2.50	< 2	< 2
	Psmb8	7.01	42.5	70.2
	Ddx58(Rig-I)	3.39	44.3	59.2
	Rnasel	-2.25	< 2	< 2
*	Rsad2(Viperin)	13.0	278	693
	Rtp4	5.24	36.2	190
	Slc15a3	8.93	2.36	2.12
	Stat1	9.15	10.9	90.3
	Stat2	2.36	5.13	6.11
	Tgtp1	4.20	180	543
	Tlr1	14.8	2.43	4.11
	Tlr2	24.1	< 2	2.52
	Tlr4	6.60	< 2	< 2
	Tlr5	2.38	< 2	< 2
	Tlr6	6.53	< 2	< 2
	Tlr7	6.65	< 2	< 2
	Tnf	13.9	< 2	< 2
	Trim25	3.44	5.26	23.3
	Trim5	2.38	2.84	< 2
	Unc93b1	8.00	< 2	< 2
*	Zbp1	4.51	34.2	33.8

*Genes selected for cloning and expression in neurons

(b) Additional candidate antiviral genes selected for cloning based on differential expression and possible immune related functions

	Gene name	Fold difference of basal level (GCN vs. CN)	Fold change after IFN-β treatment	
			GCN	CN
*	Atf3	9.45	< 2	< 2
*	Ifi204	18.1	7.58	15.7
*	Ifi205	< 2	8.66	5.73
*	Ifit2	< 2	100	5.64
*	Ifitm1	< 2	< 2	< 2
*	Ifitm2	2.30	< 2	< 2
*	Irf1	< 2	5.29	2.51
*	Irg1	115	4.22	7.75
*	Irgb10	< 2	9.48	< 2
*	Irgm1	3.05	3.79	< 2
*	Isg20	< 2	26.2	4.53
*	Lcn2	4.80	5.29	< 2
*	Mnda	24.6	18.9	9.02
*	Nmi	< 2	19.3	13.9
*	Pkr	< 2	27.2	21.5
*	Psmb9	3.30	54.3	28.8
*	Pyhin1	< 2	6.05	5.86
*	Trim12	3.32	53.4	47.9
*	Trim21	2.23	56.4	61.8
*	Trim30	11.6	24.2	459
*	Cmpk2	< 2	19.2	2.79
*	Usp18	29.4	234	3800

*Genes selected for cloning and expression in neurons.

Supplementary Table 3. Primer sequences used for qRT-PCR validation.

Gene	Forward/Reverse/Taqman probe (5' - 3')
Ifit1	GAG CCA GAA AAC CCT GAG TAC A
	AGA AAT AAA GTT GTC ATC TAA ATC
	ACT GGC TAT GCA GTC GTA GCC TAT CGC C
Rsad2	ACA CAG CCA AGA CAT CCT TC
	CAA GTA TTC ACC CCT GTC CTG
	TGT TTG AGC AGA AGC AGT CCT CGC
Oas1a	TGA GCG CCC CCC ATC T
	CAT GAC CCA GGA CAT CAA AGG
	AGG AGG TGG AGT TTG ATG TGC TG
Cmpk2	ATC CTT TAT TCC AGA AGC CCG
	CTG CGT CAG TGT GGT CTT AC
	AGT GGC ATC CAG TCC TTC AAT GGC
Stat1	GCC GAG AAC ATA CCA GAG AAT C
	GAT GTA TCC AGT TCG CTT AGG G
	ACC AAA GGA AGC ACC AGA ACC GA
Irg1	TCAGGCTCCCACCGACATAT
	GTGCCATGTGTCATCAAAATCC
	CTGCTTTTGTTAATGGTGTTGCTGTTCACTCC
Ifi27	AAT GGA GGT GGA GTT GCA G
	GAA GTG TCA TCT CCT AAG CTC AG
	TGA TTG GAG TGT GGC TAC CAG GC
Cxcl9	AATGCACGATGCTCCTGCA
	AGGTCTTTGAGGGATTTGTAGTGG
Ifi203	GAA TCA AAC AAG AGT GTA CCG TC
	TTT CTG GAA GCT GAG CCT G
Ifi204	TGG CAG CTG AGG TCT GTA AGG
	CCA GAG AGG TTC TCC CGA CTG
Ccl5	CAAGTGCTCCAATCTTGCAGTC
	TTCTCTGGGTTGGCACACAC
Cxcl5	TGG ATC CAG AAG CTC CTG TGA
	TGC ATT CCG CTT AGC TTT CTT T

SUPPLEMENTAL METHODS

***In situ* hybridization.** Brain tissues were harvested from naïve mice, and differential RNA expression was visualized by the QuantiGene View RNA *in situ* hybridization tissue assay (Affymetrix) according to the manufacturer's protocol. Briefly, paraffin-embedded tissue sections were permeabilized, gene-specific probes (*Ifi27*, *Irg1*, *Stat1*, *Ubc* (positive control), and *E.Coli Dapb* (negative control)) were hybridized, and the signal was amplified by sequential hybridization of amplifier (branched DNA). Target mRNAs were visualized by confocal microscopy after addition of the Fast Red substrate.

IFN bioassay. Levels of biologically active type I IFN in supernatants of GCN and CN cultures were determined using an encephalomyocarditis virus L929 cell cytopathic effect bioassay as described previously[1].

SDS-PAGE and immunoblot analysis. Protein lysates were harvested from wild type GCN, CN and *Stat1*$^{-/-}$ GCN in Laemmli sample buffer supplemented with 5% (v/v) β-mercaptoethanol and separated by 10% SDS-PAGE. Immunoblotting was performed using antibodies against Stat1 (Cell Signaling), β-actin (Abcam), and Rsad2/Viperin (MaP.VIP[2]).

Quantification of tissue viral burden. Infected brain tissues were harvested after extensive perfusion with phosphate-buffered saline (PBS), weighed, homogenized, and virus titers were determined by standard plaque assay on BHK21-15 cells[3].

SUPPLEMENTAL REFERENCES

1. Austin, B. A., James, C., Silverman, R. H. & Carr, D. J. J. Critical role for the oligoadenylate synthetase/RNase L pathway in response to IFN-beta during acute ocular herpes simplex virus type 1 infection. *J. Immunol.* **175**, 1100–1106 (2005).
2. Wang, X., Hinson, E. R. & Cresswell, P. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe* **2**, 96–105 (2007).
3. Diamond, M. S., Shrestha, B., Marri, A., Mahan, D. & Engle, M. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J. Virol.* **77**, 2578–2586 (2003).