Visualizing production of interferon-β by astrocytes and microglia in the brain of La Crosse virus-infected mice

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Word count abstract: 222
Word count text (excluding references, table footnotes, and figure legends): 3840

Running title: Visualizing of IFN-β in LACV-infected mouse brains

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Interferon (IFN)-β is a major component of innate immunity in mammals, but information on the in vivo source of this cytokine after pathogen infection is still scarce. To identify the cell types responsible for IFN-β production during viral encephalitis, we used reporter mice that express firefly luciferase under control of the IFN-β promoter and stained organ sections with luciferase-specific antibodies. Numerous luciferase-positive cells were detected in regions of La Crosse virus (LACV)-infected mouse brains that contained many infected cells. Double staining experiments with cell-type specific markers revealed that similar numbers of astrocytes and microglia of infected brains were luciferase-positive, whereas virus-infected neurons rarely contained detectable levels of luciferase. Interestingly, if a mutant LACV unable of synthesizing the IFN-antagonistic factor NSs was used for challenge, the vast majority of the IFN-β-producing cells in infected brains were astrocytes rather than microglia. Similar conclusions were reached in a second series of experiments in which conditional reporter mice expressing the luciferase reporter gene solely in defined cell types were infected with wild-type or mutant LACV. Collectively, our data suggest that glial cells rather than infected neurons represent the major source of IFN-β in LACV-infected mouse brains. They further indicate that IFN-β synthesis in astrocytes and microglia is differentially affected by the viral IFN antagonist, presumably due to differences in LACV susceptibility of these two cell types.
**Introduction**

Viruses can trigger pattern recognition receptors of infected hosts which initiate signaling cascades that culminate in transcriptional activation of type I and type III interferon (IFN) genes. Type I and type III IFNs are cytokines that use distinct receptor complexes for signaling and which, thereby, induce an antiviral state in uninfected cells. The family of type I IFN includes more than ten different IFN-α subtypes, IFN-β and minor subtypes such as IFN-ω or IFN-δ, whereas type III IFN includes IFN-λ1, -λ2 and -λ3 (8, 19). These various IFN genes are typically co-induced in response to virus infection, although the kinetics and the degree of activation of the different IFN genes differ considerably depending on producer cell type and nature of challenge virus (6, 15). In the mouse, IFN-β is the first type I IFN subtype being expressed after viral infection and, together with IFN-α4, is considered to prime cells for the production of other type I IFN family members (2).

Pattern recognition receptors which can recognize RNA viruses include cytoplasmic RIG-like helicases and membrane-anchored toll-like receptors (TLR). Cell culture studies indicate that most if not all nucleated mammalian cells can synthesize IFN in response to signals from RIG-like helicases when infected with replication-competent viruses (21). Further, certain immune cells such as macrophages and dendritic cells readily synthesize IFN when receiving signals from TLRs which recognize engulfed virus-derived nucleic acids (14). The situation after infection of an intact organism is much more complex. For viruses that cause viremia, plasmacytoid dendritic cells (pDC) which are mainly present in blood and spleen of mammals are responsible for most of the circulating IFN (1, 7, 24). During influenza virus infection of the lung, pDC seem to play far less important roles (12). Similarly, classical immune cells including pDCs are not present in healthy brains (10, 13), suggesting that other cell types are mainly responsible for IFN synthesis in this organ. However, previous attempts to unambiguously identify these alternative IFN-producing cells did not yield a clear picture. Experiments were either performed with isolated brain cells or were focused on certain cell types without addressing the question of the contribution of such cells to the overall IFN-response in the central nervous system (10, 22, 23, 26). One difficulty with these experimental approaches was that IFNs are quickly secreted and are not accumulating to high intracellular levels in producer cells, thus complicating their detection in tissue slices by IFN-specific antibodies.

La Crosse virus (LACV) is a mosquito-borne pathogen that infects up to 300,000 people in the United States and can cause encephalitis in children and young adults (3). LACV belongs to the genus *Orthobunyavirus*, family *Bunyaviridae*. These viruses have a tri-segmented
single-stranded RNA genome of negative polarity and replicate in the cytoplasm of infected cells. The smallest genome segment of LACV codes for the viral nucleoprotein and a non-structural protein, termed NSs, which efficiently inhibits the IFN system of infected mammalian hosts. NSs of LACV induces degradation of cellular RNA polymerase II which, in turn, results in reduced transcription of many cellular genes, including the genes for type I and type III IFN (27). A mutant of LACV lacking a functional NSs gene (LACV-ΔNSs) was generated (5). As expected if NSs served as IFN antagonist, LACV-ΔNSs induced significantly more IFN in cultured cells and brains of infected mice than wild-type LACV (4, 18). Further, the LACV-ΔNSs mutant was less virulent than wild-type LACV in mice, although both viruses cause encephalitis and death after 5-10 days if administered intraperitoneally into juvenile mice (4). Using conventional in situ hybridization and immunostaining techniques, we previously identified cells with macrophage and ependymal markers as major sources of IFN-α and -β in the brain of mice with acute LACV encephalitis, and we observed that neurons represent a minor but substantial source of IFN during viral encephalitis (10).

Transgenic mice in which reporter genes were inserted into the coding regions of the IFN-α or -β genes are promising new tools for studying virus-induced expression of IFN genes in vivo. A reporter mouse which expresses green-fluorescent protein (GFP) in place of IFN-α6 was successfully used to demonstrate that alveolar macrophages contribute to IFN synthesis in virus-infected lungs (17). Cells from a reporter mouse with a modified IFN-β locus encoding GFP were used to demonstrate the stochastic nature of type I IFN gene expression (28). We recently employed another reporter mouse in which the IFN-β coding region is replaced by luciferase to visualize IFN synthesis in virus-infected animals by in vivo imaging (18). We now used the same luciferase reporter mouse to analyze the contribution of various brain cell types to IFN-β synthesis in mice with LACV encephalitis. We visualized IFN-β-producing cells by staining brain sections with antibodies that simultaneously recognize luciferase and marker proteins of neurons, astrocytes and microglia, respectively. We further took advantage of the possibility that the loxP-flanked luciferase gene in this reporter mouse can be rearranged in defined cell types by crossing these animals with mice that express Cre recombinase in a cell type-specific manner. Using these two approaches we identified astrocytes and microglia as the main IFN-β producers in LACV-infected brains. We further observed that the LACV-encoded IFN-antagonistic factor NSs strongly impairs IFN production by astrocytes but not microglia.
Materials and Methods

Mice: Mice were bred in the animal facility of the Department of Virology at the University of Freiburg. All mice used in this study were on the C57BL/6 background, or backcrossed onto C57BL/6 for at least 5 generations. IFN-β<sup>+/Δβ-luc</sup> and conditional reporter IFN-β<sup>+/floxed-β-luc</sup> mice have been described previously (18, 25). IFN-β<sup>flox-β-luc</sup> mice were crossed to LysM-Cre (25), Thy1-Cre (11) (Jackson Lab, Stock Number 006143) and Synapsin1-Cre mice (29) to generate mice that express the reporter gene either in microglia and macrophages, astrocytes and neurons, and neurons, respectively.

Viruses and infection of mice: Wild-type LACV and mutant LACV-ΔNSs that cannot express the IFN antagonistic factor NSs were previously described (5). Virus stocks were generated in Vero cells. Juvenile mice (12-15 days old) were infected intraperitoneally with 10<sup>4</sup> plaque forming units (PFU) of wild-type or mutant LACV diluted in 100 µl of phosphate buffer saline (PBS). Animals were checked for neurological symptoms at 8 h intervals and sacrificed when first signs of ataxia were noted. Brains were either collected without fixation for virus titration and measuring luciferase activity or were perfusion fixed for immunohistochemical analyses as described below.

Virus titrations: Plaque assays were performed in Vero cells using 6-well plates. Serial dilutions of lysates were applied for 1 h. Supernatants were then removed and replaced by a 1:1 mixture of 3% Avicel-cellulose (FMC BioPolymer) and double concentrated DMEM (Gibco). Vero cells were incubated for 72 h at 37 °C and 5% CO<sub>2</sub> before supernatants were removed. Cells were fixed with 4% paraformaldehyde, and plaques were visualized by staining with 0.5% crystal violet.

Ex vivo luciferase measurement: Brains homogenized in 800 µl of PBS using the FastPrep-24 equipment and Lysing Matrix A (MP Biomedicals). Samples (200 µl) were treated with 50 µl of 5x Cell Culture Lysis Buffer (Promega), and luciferase activity was measured in a Sirius Tube Luminometer (Berthold Technologies) using the single Luciferase Assay System (Promega) according to the manufacturer’s protocol.

Immunohistochemistry: Animals were sacrificed with a mixture of ketamine (3.7%), xylazine (0.2%) and acepromacine (0.02%) and transcardially perfused with 0.9% NaCl followed by 4% buffered paraformaldehyde in PBS. Brains were postfixed in the same solution for 6 more
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hours. Fixed brains were cut horizontally into 50-μm-thick sections on a Leica Vibratome. Free-floating tissue sections were blocked and permeabilized in PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 30 min. Sections were then incubated with rabbit anti-luciferase antibody (Fitzgerald, 70C-CR2020RAP), mouse anti-NeuN (Millipore, MAB377), rat anti-F4/80 (AbD Serotec, MCA497R), mouse anti-GFAP (SIGMA-ALDRICH, G3893) or mouse anti-LACV-G2 (QED Bioscience, 18752) in PBS containing 3% normal donkey serum at 4 °C overnight. For detection of luciferase, signal amplification with the TSA Fluorescein System (PerkinElmer) was performed according to the manufacturer's instructions using a biotin-conjugated donkey anti-rabbit antibody (Jackson Immunoresearch). For cellular markers, appropriate DyLight488-, DyLight549-, Cy3-, or Cy2-conjugated secondary antibodies (Jackson Immunoresearch) were used. Slides were mounted in DAPI-containing IS Mounting Medium (Dianova). Digital images were taken with an ApoTome fluorescence microscope (Zeiss) using AxioVision software.

Results

Identification of IFN-β-producing cells in LACV-infected brains

To visualize IFN-β-producing cells in the brain of LACV-infected heterozygous reporter mice, we stained tissue slices from animals exhibiting clinically apparent encephalitis with luciferase-specific antibodies. Because luciferase expression levels were expected to be low and paraffin- or cryo-embedding might affect epitope recognition, we decided to work exclusively with paraformaldehyde-fixed free floating slices prepared by vibratome sectioning. We observed that luciferase signals were very faint if standard histological staining techniques were applied. Consequently, signal amplification was routinely employed for better visualization of IFN-β-producing cells.

Luciferase-positive cells were typically observed in distinct clusters which were present in all parts of virus-infected brains. Double-staining experiments revealed that the luciferase-positive cell clusters exclusively mapped to brain regions in which virus-infected cells were highly abundant (Fig. 1). As expected from the fact that LACV encodes the IFN-antagonist factor NSs, the number of luciferase-positive cells was at least 10-fold higher in brains of diseased mice infected with the LACV-ΔNSs mutant (Fig. 1A-D) compared to brains infected with LACV-wt (Fig. 1E-H). Detailed inspection revealed that although mostly found in close proximity to virus-infected cells, luciferase-positive cells were usually not positive for viral
antigen (Fig. 1B, 1F, 1D and 1H). It was shown previously that LACV predominantly infects neurons (16). Shape and distribution of luciferase-positive cells indicated that they might be mostly astrocytes. This was particularly obvious in the cerebellum, where luciferase-positive Bergmann glia cells were typically observed in immediate vicinity of virus-infected Purkinje cell somata (Fig. 1C and 1D).

Majority of IFN-β-producing cells in LACV-infected brains are astrocytes and microglia

To assess the extent to which the various cell types in LACV-infected brains might contribute to IFN-β synthesis, we determined which fractions of luciferase-positive cells could unambiguously be classified as astrocytes, neurons or microglia by double-staining with antibodies that recognize cell type-specific markers. This analysis was performed with both wild-type LACV and the NSs-deficient mutant virus to address the question of whether the IFN-antagonistic factor NSs might act predominantly in certain cell types. In agreement with previous results (4), we found that luciferase levels in brains of mice infected with the ΔNSs virus were about 6-fold enhanced, although the mutant virus replicated substantially less well than wild-type virus with about 30-fold reduced peak brain titers (Fig. 2). A large number of luciferase-positive cells in brains of mice infected with wild-type or NSs-deficient LACV expressed GFAP, indicating that they represent astrocytes (Fig. 3A and 3B). Another fraction of luciferase-positive cells expressed the macrophage/microglia marker protein F4/80 (Fig. 3C and 3D). A very small proportion of luciferase-expressing cells stained positive for the neuron marker NeuN (Fig. 3E). Interestingly, luciferase-positive neurons were detected at low frequency in brains of mice infected with LACV-ΔNSs (Fig. 3E) but not LACV-wt (Fig. 3F).

To make this analysis more quantitative, we compiled the results from a detailed inspection of tissue slices from three or more severely diseased animals per virus strain. At least 300 luciferase-positive cells were evaluated individually for each double-staining experiment listed in table 1. In brains of mice infected with the LACV-ΔNSs mutant, the vast majority (89%) of luciferase-positive cells expressed GFAP, suggesting that they represent astrocytes. The F4/80 marker which is present on microglia and infiltrating macrophages was expressed by approximately 5% of the luciferase-positive cells in brains of mice infected with LACV-ΔNSs. The number of luciferase-positive cells expressing NeuN was approximately 1%, suggesting that only very few neurons can synthesize large amounts of IFN-β. A strikingly different picture emerged when the staining data from wild-type LACV-infected mice were compiled. In this case, the frequency of luciferase-positive astrocytes was only 35%, whereas the frequency of luciferase-positive microglia/macrophages was 62% (Table 1). No
luciferase-positive cells expressing NeuN were detected in brains which were infected with wild-type LACV.

LACV replicates predominantly in neurons

As the majority of IFN-producing cells were seemingly uninfected astrocytes and microglia, we carefully analyzed if LACV can establish productive infections in those cell types. To do this, we subjected brains infected with LACV-wt or LACV-ΔNSs to simultaneous staining for viral antigen and specific markers for neurons, astrocytes or microglia. In line with previous data (16), we found that LACV-wt and LACV-ΔNSs replicated almost exclusively in neurons (Fig 4A and 4B). We could not detect any virus-positive microglia (data not shown), and less than 1% of the virus-positive cells in the infected brains were astrocytes (Fig. 4C and 4D). Thus, astrocytes are susceptible for LACV, although infection of such cells might mostly be non-productive.

Luciferase production in virus-infected conditional reporter mice

To confirm the IFN-β expression patterns observed by immunohistochemistry, we created reporter mice in which luciferase is expressed exclusively in predetermined cell types. Such animals may be generated by breeding reporter mice which contain strategically positioned loxP sites with mice that selectively express Cre recombinase in defined cell types (25). For the current study we used Synapsin1-Cre mice to generate animals in which expression of luciferase is restricted to neurons (29). Further, we used Thy1-Cre mice to produce reporter mice which express luciferase in both neurons and astrocytes (11). Finally, we employed LysM-Cre mice to generate reporter mice in which luciferase expression is restricted to microglia and macrophages (9, 20).

To verify the predicted luciferase gene expression patterns in our conditional reporter mice we performed double-staining experiments of brain sections from LACV-ΔNSs-infected LysM-Cre, Thy1-Cre or Synapsin-Cre reporter mice with antibodies recognizing luciferase and the corresponding markers F4/80, GFAP and NeuN. As expected if the reporter mice expressed the luciferase gene with desired cell type specificity, we found that luciferase-positive cells in brains of infected LysM-Cre mice expressed F4/80, an antigen of microglia and macrophages (Fig. 5A), but not the astrocyte marker GFAP (data not shown). Similarly, the luciferase-positive cells observed in brains of Thy1-Cre mice expressed GFAP (Fig. 5B), but not F4/80 (data not shown). Further, the few luciferase-positive cells that we detected in Synapsin-Cre reporter mice expressed the neuron marker NeuN (Fig. 5C). Thus, the conditional reporter...
mice used here represent suitable tools for the assessment of the relative contributions of the various cell types to overall IFN synthesis in LACV-infected brains. Since luciferase expression is driven by the virus-inducible IFN-β promoter in our mice, a direct comparison of reporter gene activity in individual animals will only yield meaningful quantitative data if virus replication in brains is similar. Although the different conditional reporter mice showed only slight differences in onset of symptoms or course of disease (Fig. 6), we tried to minimize errors resulting from such variation by restricting this comparison to animals containing matching viral brain titers (~8x10^6 pfu in the case of LACV-ΔNSs and ~1x10^8 pfu in the case of LACV-wt). Under these experimental conditions, “global” Δβ-luc mice infected with LACV-ΔNSs contained, at average, 5x10^5 RLU of luciferase activity per µl of brain extract (Fig. 7A). Luciferase activity in brain extracts from Thy1-Cre mice infected with LACV-ΔNSs was only slightly reduced, suggesting that at least 71.4% of the luciferase signal originates from virus-mediated stimulation of astrocytes and neurons (Fig. 7B). Luciferase activity in extracts from brains of Synapsin1-Cre mice was 9.4% of “global” mice, whereas luciferase activity in extracts of brains from LysM-Cre mice infected with LACV-ΔNSs was comparatively low. If compared to “global” mice, the signal in LysM-Cre mice was only about 1.7% at average (Fig. 7B). Thus, astrocytes, neurons and microglia together accounted for ~75% of luciferase activity of “global” reporter mice. The cellular origin of the missing ~25% of activity (white sector in Fig. 7B) remains unclear. It most likely indicates incomplete Cre-mediated recombination of the lox-P-tagged target gene in our mice. A different picture emerged when LACV-wt was used for the infection study. First, as discussed above, luciferase activity in brain extracts of “global” mice infected with LAVC-wt was about 6-fold lower than in “global” mice infected with LACV-ΔNSs and, at average, reached values of only 9x10^4 RLU per µl of brain extract (Fig. 7C). Second, compared to infection with LACV-ΔNSs, luciferase activity in brains of wild-type LACV-infected LysM-Cre mice was significantly increased. At average, it accounted for 41.4% of the signal observed in “global” mice infected with wild-type LACV (Fig. 7D). Third, the contribution of neurons to overall luciferase activity did not differ substantially in mice infected with either wild-type or mutant LACV, whereas the contribution of astrocytes was clearly less prominent in wild-type LACV-infected mice compared to mice infected with the ΔNSs mutant virus (Fig. 7D). Thus, our experiments with the conditional reporter mice could confirm the conclusions from our double-staining experiments with the “global” reporter mice which suggested that the two virus variants induce a strikingly different cellular IFN-β expression pattern.
Discussion

Employing transgenic mice that express a luciferase reporter gene under control of the IFN-β promoter we established a staining protocol that can identify single IFN-β-producing cells in virus-infected brain tissue. To quantify the relative contribution of specific cell types to overall IFN-β synthesis in the brain, we took advantage of the Cre-Lox system and generated reporter mice that express the luciferase transgene either in astrocytes and neurons, neurons only, or microglia and macrophages. When using La Crosse virus as a model for viral encephalitis, we found that astrocytes and microglia were the main producers of IFN-β in the infected brain, whereas the contribution of infected neurons was relatively small. Interestingly, when a mutant virus was used for challenge that cannot synthesize the IFN-antagonistic factor NSs, the balance was shifted and astrocytes became the dominant IFN-β producers. Our work demonstrates that besides infected cells, seemingly uninfected cells also contribute massively to IFN synthesis in the central nervous system. Our work further demonstrates that virus-encoded antagonistic factors can affect IFN production by acting selectively on distinct cell types.

Previous attempts to characterize the production of IFN during viral encephalitis by in situ hybridization technology had already indicated that various cell types, including neurons, contain detectable levels of type I IFN (10). These authors had used specific antisera to visualize IFN-α- and IFN-β-producing cells in virus-infected brains. However, signals were weak, and quantitative analyses were depended on visual interpretation of histological data. This difficulty presumably originates from the fact that type I IFN is quickly secreted from producer cells and fails to accumulate to high intracellular levels, thus complicating detection by immunostaining. The reporter mice that we used in this study overcome this problem as the luciferase molecule lacks export signals and thus accumulates in the cytoplasm of the producer cells. Nevertheless, staining of tissue for luciferase in virus-infected reporter mice was challenging as standard histological protocols failed to produce detectable signals. We could eventually overcome these problems by using mild conditions for sectioning and staining, and by including a signal amplification step.

Our immunostaining approach is backed-up well by results from experiments with conditional reporter mice that express the IFN-β-promoter-regulated luciferase gene exclusively in defined cell types. We stringently evaluated the specificity of Cre-mediated recombination in these mice and excluded the possibility that reporter gene expression by undesired cell types clouded the picture. As predicted, LysM promoter-driven expression of Cre recombinase seemed to rearrange the IFN-β locus exclusively in cells that were positive for the
microglia/macrophage marker F4/80. Similarly, Thy1 promoter-driven expression of Cre recombinase seemed to activate the loxP-tagged reporter gene only in astrocytes and presumably neurons. Synapsin1-driven expression of Cre recombinase resulted in selective rearrangement of the loxP-tagged IFN-β locus in neurons.

The results of our immunostaining experiments and those of our Cre-loxP approach showed a very good correlation in the case of astrocytes and microglia, but no clear correlation in the case of neurons. This discrepancy can easily be explained by the high detection threshold of the immunostaining technique. Most likely, the IFN-β-promoter-driven luciferase gene got activated to a low extent in LACV-infected neurons, but luciferase levels in individual cells remained too low for detection by antibody staining. Since a large percentage of neurons got productively infected with LACV in our mice, it is likely that the small contribution of individual neurons did add up considerably. These considerations may explain why experiments with our Synapsin1-Cre reporter mice indicated a more substantial contribution of neurons to luciferase activity in LACV-infected brains than the histological analysis.

A remarkable finding of our study was that astrocytes contribute substantially to IFN synthesis in the virus-infected brain. This result was not expected, as we (Fig. 4 A and 4B) and others (4, 10, 16) showed that productive replication of LACV is largely restricted to neurons. Since the contribution of astrocytes was much more pronounced if mutant LACV was used for infection that cannot synthesize the IFN-antagonistic factor NSs, we assume that a high number of brain astrocytes actually got infected by LACV, although such infections usually remained non-productive. In line with this hypothesis, our immunofluorescence analysis (Fig. 4C and 4D) demonstrated that LACV can indeed establish productive infection of astrocytes, although this may occur only rarely.

We noted that the luciferase signal in microglia was not negatively regulated by NSs. In fact, IFN production by microglia was even higher if wild-type virus was administered instead of LACV-ΔNSs. Therefore, IFN production by microglia correlated directly with the virus load in the brain, irrespective of whether the challenge virus coded for NSs or not. Since the two viruses used here differ slightly with regard to kinetics of disease induction, it remains possible that the kinetics of virus-induced cytokine gene activation in astrocytes and microglia also differed somewhat and thus contributed to the difference which we observed between LACV-wt and LACV-ΔNSs. To minimize variations of this sort, we restricted our analysis to animals which showed similar signs of neurological disease. Taking all these caveats into account, the most coherent explanation of the various observations reported here seems that, in contrast to astrocytes, IFN production by microglia is not triggered by intracellular virus
sensors such as Rig-I but rather by alternative sensors such as TLR which can detect viral components in the extracellular space. Interestingly, recent LACV infection experiments with TLR7-deficient mice failed to reveal a prominent role of this virus sensor in our system (unpublished data), suggesting that several virus sensors might get activated simultaneously in the LACV-infected mouse brain.

Taken together, our study demonstrates that three cell types are responsible for the bulk of IFN synthesis during acute encephalitis after LACV infection, namely productively infected neurons, abortively infected astrocytes and uninfected microglia. Interestingly, if not inhibited by the viral IFN-antagonistic factor NSs, the amount of IFN-β expressed by neurons exceeds the quantity of IFN-β produced by microglia and macrophages. In line with these findings, IFN synthesis by astrocytes was also strongly inhibited by the viral IFN-antagonistic factor NSs, whereas microglia proved to be a more robust source of IFN synthesis.

**Acknowledgement**

We thank Thomas Michiels and Marco Prinz for helpful discussions, and Alexandra Sontheimer and Anke Augspach for help with mouse genotyping.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 620).

**References**


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Figure Legends

Figure 1: IFN-β-producing cells are found near LACV-infected cells in various brain regions. Brain sections from cortex (A, B, E, F) and cerebellum (C, D, G, H) of diseased IFN-β^{+/Δβ-luc} mice infected with LACV-ΔNSs (A, B, C, D) or LACV-wt (E, F, G, H) were simultaneously stained for luciferase (green), viral antigen (LACV-G2, red) and cell nuclei (DAPI, blue). Note that although in close proximity to infected cells, luciferase-positive cells did usually not stain for viral antigen. Size bars: 50 µm.

Figure 2: LACV-ΔNSs activates the IFN-β reporter gene more efficiently than LACV-wt, although it replicates less well in the brain. Brains of infected IFN-β^{+/Δβ-luc} mice showing signs of neurological symptoms were collected and homogenized. Viral titers (A) and luciferase activity (B) in brain homogenates were determined. *** p< 0.0005

Figure 3: Astrocytes, microglia and neurons of LACV-infected mouse brains express IFN-β reporter gene. Double-staining of brain slices for luciferase (luc) and astrocyte marker GFAP (A, B), microglia/macrophage marker F4/80 (C, D) and neuron marker NeuN (E, F). Luciferase positive neurons were detectable only in LACV-ΔNSs (E) but not in LACV infected brains (F). Cells were counterstained with DAPI. Single channel and merged pictures of the same frames are shown. Size bars: 10 µm.

Figure 4: LACV replicates predominantly in neurons. Brains of mice infected with LACV-ΔNSs (A, C) or LACV-wt (B, D) were stained simultaneously for viral antigen (LACV-G2, green) and either the neuron marker NeuN (red in panels A and B) or the astrocyte marker GFAP (red in panels C and D). Size bars: 20 µm.

Figure 5: Conditional reporter mice express the luciferase reporter gene in the predicted cell types. (A) LysM-Cre^{+/}-IFN-β^{+/floxβ-luc} mice were infected with LACV-ΔNSs and brains of diseased mice were simultaneously stained for luciferase (green) and the microglia/macrophage marker F4/80 (red). (B) Thy1-Cre^{+/}-IFN-β^{+/floxβ-luc} mice were infected with LACV-ΔNSs, and brains of diseased mice were simultaneously stained for luciferase (green) and the astrocyte marker GFAP (red). (C) Synapsin1-Cre^{+/}-IFN-β^{+/floxβ-luc} mice...
infected with LACV-ΔNSs and brain sections of diseased mice were simultaneously stained for luciferase (green) and the neuron marker NeuN (red). Size bars: 50 µm.

**Figure 6**: Conditional reporter mice show no significant differences in susceptibility to LACV. “Global” reporter mice (Δβ-luc) and conditional Thy1-Cre<sup>+/--</sup>IFN-β<sup>+/flox</sup>-luc (thy), LysM-Cre<sup>+/--</sup>IFN-β<sup>+/flox</sup>-luc (lys) and Synapsin1-Cre<sup>+/--</sup>IFN-β<sup>+/flox</sup>-luc (syn) reporter mice infected with LACV-ΔNSs (A) or LACV-wt (B) were monitored for neurological symptoms. Diseased animals were killed and brain titers were determined. No significant differences were observed between the different mouse strains.

**Figure 7**: IFN-β synthesis by astrocytes and neurons but not microglia is repressed by LACV-encoded IFN-antagonistic factor NSs. Reporter mice in which the luciferase gene is expressed exclusively in astrocytes and neurons (thy), neurons only (syn) or microglia/macrophages (lysM) were infected with LACV-ΔNSs (A, B) or LACV-wt (C, D). “Global” IFN-β<sup>+/Δβ-luc</sup> reporter mice (Δluc) served as reference. Brains of diseased animals with very similar virus load were selected for further analysis. Mean luciferase activities (with standard deviation) in brain samples from the various mouse strains infected with either LACV-ΔNSs (A) or LACV-wt (C) are shown. The average contributions of different cell types to luciferase activity in brains of mice infected with LACV-ΔNSs (B) or LACV-wt (D) are shown as pie charts, in which the activity of “global” IFN-β<sup>+/Δβ-luc</sup> reporter mice was set to 100%.
Table 1: Frequency of luciferase-positive astrocytes, microglia/macrophages and neurons in mouse brains infected with wild-type or mutant LACV

<table>
<thead>
<tr>
<th>Virus</th>
<th>Astrocytes a)</th>
<th>Microglia/Macrophages b)</th>
<th>Neurons c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACV-ΔNSs</td>
<td>89% (536/603)</td>
<td>5% (29/561)</td>
<td>1% (4/424)</td>
</tr>
<tr>
<td>LACV-wt</td>
<td>35% (134/379)</td>
<td>62% (189/306)</td>
<td>0% (0/337)</td>
</tr>
</tbody>
</table>

a) Astrocytes are defined here as GFAP-positive cells
b) The microglia/macrophage population is defined here as F4/80-positive cells
c) Neurons are defined here as NeuN-positive cells
d) Cells positive for corresponding marker/number of luciferase-positive cells analyzed