

# Virus-Induced Transcriptional Changes in the Brain Include the Differential Expression of Genes Associated with Interferon, Apoptosis, Interleukin 17 Receptor A, and Glutamate Signaling as Well as Flavivirus-Specific Upregulation of tRNA Synthetases

Penny Clarke,<sup>a</sup> J. Smith Leser,<sup>a</sup> Richard A. Bowen,<sup>d</sup> Kenneth L. Tyler<sup>a,b,c,e</sup>

Departments of Neurology,<sup>a</sup> Medicine,<sup>b</sup> and Microbiology,<sup>c</sup> University of Colorado School of Medicine, Aurora, Colorado, USA; Department of Biomedical Sciences, Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado, USA<sup>d</sup>; Denver Veterans Administration, Denver, Colorado, USA<sup>e</sup>

**ABSTRACT** Flaviviruses, particularly Japanese encephalitis virus (JEV) and West Nile virus (WNV), are important causes of virus-induced central nervous system (CNS) disease in humans. We used microarray analysis to identify cellular genes that are differentially regulated following infection of the brain with JEV (P3) or WNV (New York 99). Gene expression data for these flaviviruses were compared to those obtained following infection of the brain with reovirus (type 3 Dearing), an unrelated neurotropic virus. We found that a large number of genes were up-regulated by all three viruses (using the criteria of a change of >2-fold and a *P* value of <0.001), including genes associated with interferon signaling, the immune system, inflammation, and cell death/survival signaling. In addition, genes associated with glutamate signaling were down-regulated in infections with all three viruses (criteria, a >2-fold change and a *P* value of <0.001). These genes may serve as broad-spectrum therapeutic targets for virus-induced CNS disease. A distinct set of genes were up-regulated following flavivirus infection but not following infection with reovirus. These genes were associated with tRNA charging and may serve as therapeutic targets for flavivirus-induced CNS disease.

**IMPORTANCE** Viral infections of the central nervous system (CNS) are an important cause of morbidity and mortality. Treatment options for virus-induced CNS disease are limited, and for many clinically important neurotropic viruses, no specific therapy of proven benefit is currently available. We performed microarray analysis to identify genes that are differentially regulated in the brain following virus infection in order to identify pathways that might provide novel therapeutic targets for virus-induced CNS disease. Although several studies have described gene expression changes following virus infection of the brain, this report is the first to directly compare large-scale gene expression data from different viruses. We identified genes that are differentially regulated in infection of the brain with viruses from different families and those which appear to be specific to flavivirus infections.

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Address correspondence to Penny Clarke, Penny.Clarke@ucdenver.edu.

Vector-transmitted arboviral diseases are a significant, and increasing, cause of human disease (reviewed by Tyler [1]). Many clinically important arboviral infections are caused by members of the genus *Flavivirus* of the *Flaviviridae*, including Japanese encephalitis virus (JEV), the most significant cause of mosquito-borne encephalitis worldwide, and West Nile virus (WNV), the leading cause of epidemic encephalitis in the United States. Flaviviruses circulate in transmission cycles consisting of vertebrate hosts and insect vectors, with humans acting as “dead-end” hosts. The most devastating outcome of infection with JEV or WNV is neuroinvasive disease, which can include encephalitis, meningitis, or myelitis. Although neuroinvasive disease occurs in <1% of JEV- and WNV-infected individuals, both viruses readily induce encephalitis in mice, where neuropathology replicates the essential features of serious human disease.

There are many similarities between the CNS diseases induced by JEV and WNV. Both viruses primarily affect gray matter and show a predilection for specific areas, including the basal ganglia, cerebellum, and spinal cord (2–9). Histologically, CNS disease induced by both viruses is characterized by inflammatory infiltrates, necrotic foci, astrogliosis, and the formation of microglial nodules. In the CNS, neurons, particularly pyramidal neurons (JEV and WNV) and cerebellar Purkinje cells (WNV), constitute the main cellular target for both viruses. However, infection of vascular endothelial and ependymal cells (JEV), astrocytes (JEV and WNV), and monocytes (JEV and WNV) has been reported (2, 3, 7–11). Japanese encephalitis virus has a particular tropism for developing neurons and neuroprogenitor cells, which might explain the predilection of JEV for specific brain regions as well as the severity of JEV infections in children. In contrast, WNV neu-

roinvasive disease is largely a disease of elderly persons, although the exact mechanisms responsible remain poorly understood. There are no specific therapies for JEV- or WNV-induced CNS disease, and treatment remains supportive (12). An effective vaccine exists for JEV; however, widespread implementation in high-risk areas has proven difficult.

The neuropathogenic mechanisms that bring about JEV- and WNV-induced neuronal death remain poorly understood. We used large-scale gene expression arrays to identify and compare cellular genes and signaling pathways that are activated in the mouse brain following infection with JEV and WNV. These genes and signaling pathways may play a role in flavivirus pathogenesis within the CNS and serve as potential therapeutic targets for treatment of flavivirus-induced CNS disease. Although several studies have analyzed large-scale gene expression changes following JEV and WNV infection, at both the transcriptional and translational levels, no studies have directly compared the expression profiles of the two viruses in a consistent experimental model system (13–18). Flavivirus-induced changes in gene expression were compared to gene expression induced in the mouse brain following infection with Type 3 Dearing (T3D) reovirus, an unrelated neurotropic virus, to identify additional therapeutic targets which may have broad-spectrum applicability to neurotropic viruses. Reovirus infection of neonatal mice is a classic model of viral pathogenesis that has been widely used as a model for viral encephalitis (19). In the CNS, T3D reovirus targets neurons in the cortex, hippocampus, thalamus, and spinal cord. In contrast to flavivirus infections, reovirus-induced CNS disease is not characterized by inflammatory infiltrates, and although gliosis is induced following T3D reovirus infection, this does not appear to be due to infection of either astrocytes or microglia (20). Our laboratory has previously used Affymetrix microarrays to identify genes that are differentially regulated following infection of mice with reovirus (21).

## RESULTS

**Flavivirus infection of the brain leads to the differential expression of cellular genes.** Affymetrix 1.0 mouse whole-genome chips were used to compare gene expression changes in the brain following infection with JEV and WNV. Adult Swiss Webster (SW) mice were infected with 40 PFU JEV or 100 PFU WNV by intracerebral (i.c.) inoculation. These doses were chosen to induce similar progression of virus-induced CNS disease. Five to six days following infection, when mice were showing clinical signs of encephalitis, brains were removed and gene expression changes were analyzed (Fig. 1A). Principal components analysis indicated that for each virus, mock-infected and infected brains show divergent gene expression profiles. In contrast, there was striking similarity between the gene expression profiles of individual mock-infected and infected mice (Fig. 1B). In addition, the gene expression profiles were more similar between animals infected with different flaviviruses than between animals infected with flaviviruses and those infected with reovirus (Fig. 1B). Six hundred fourteen genes were found to be up-regulated (criteria, a change of  $>2$ -fold and a  $P$  value of  $<0.001$ ) in the brain following infection with JEV, and 582 genes were up-regulated (criteria, a change of  $>2$ -fold and a  $P$  value of  $<0.001$ ) following infection with WNV (Fig. 1C; also, see Tables S1 and S2 in the supplemental material). Three hundred fifty-two up-regulated genes were common to infections with JEV and WNV, indicating that 60% (352/582) of genes that are up-regulated in the brain following infection with WNV are also up-

**TABLE 1** Function of genes up-regulated in the brain following virus infection<sup>a</sup>

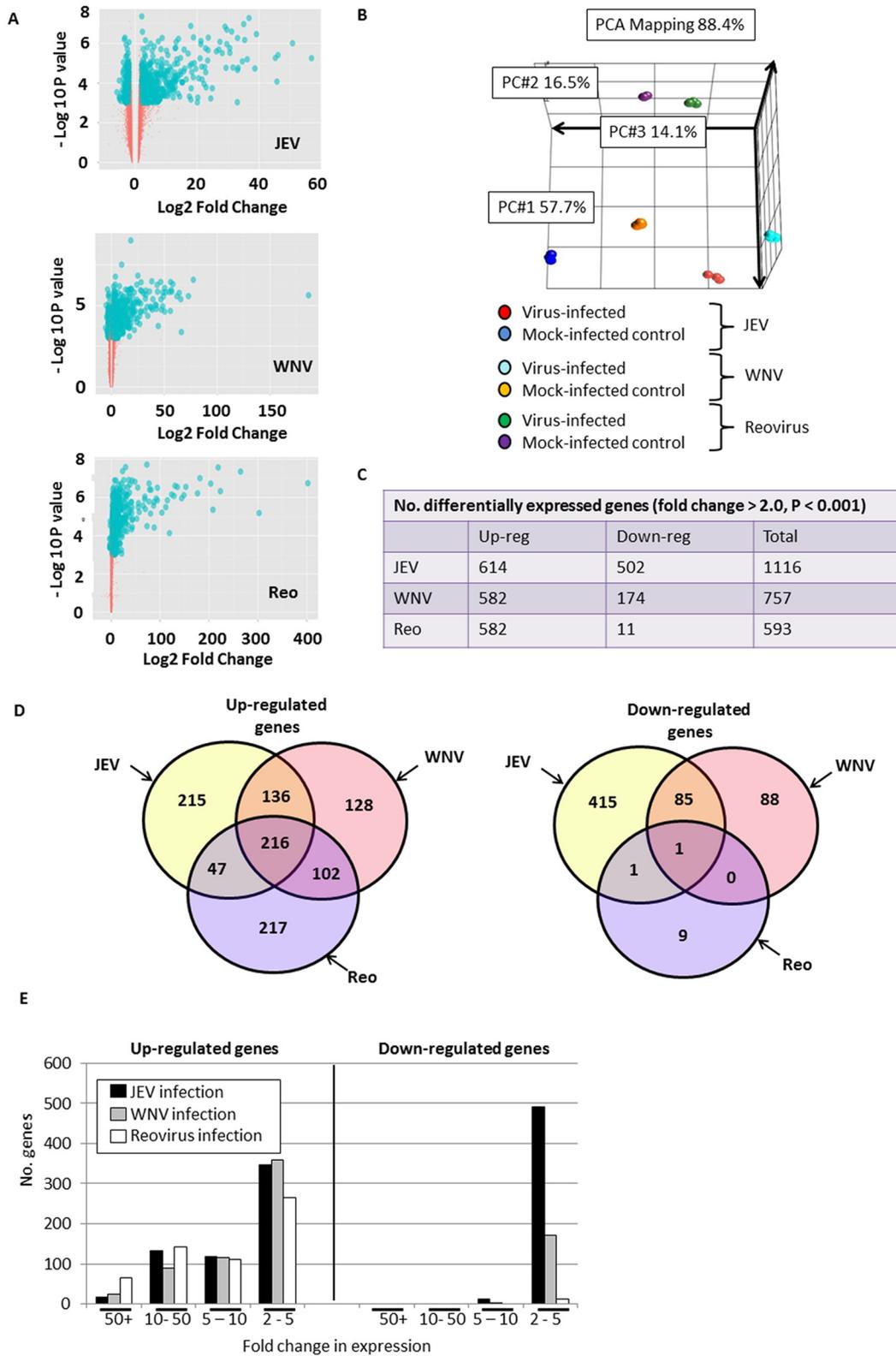
| General function                        | No. of genes |
|---|--------------|
| IFN signaling                           | 50           |
| Immune system                           | 40           |
| Inflammation/acute phase response       | 22           |
| Acute phase response                    | 8            |
| TLR signaling                           | 4            |
| Apoptosis/cell death                    | 17           |
| Regulation of transcription/translation | 9            |
| Tumorigenesis                           | 3            |
| Retinoic acid signaling                 | 2            |
| Lipid metabolism                        | 4            |
| Cell growth/development                 | 2            |
| Transport                               | 4            |
| Cell structure                          | 2            |
| Nucleic acid editing/repair/synthesis   | 5            |
| Undefined function                      | 44           |
| Total                                   | 216          |

<sup>a</sup> Mice were infected with JEV, WNV or reovirus. At 5 to 8 days postinfection brains were harvested and RNA was analyzed using Affymetrix microarrays. Two hundred sixteen genes were upregulated in infections with the 3 viruses (change,  $>2$ -fold;  $P < 0.001$ ).

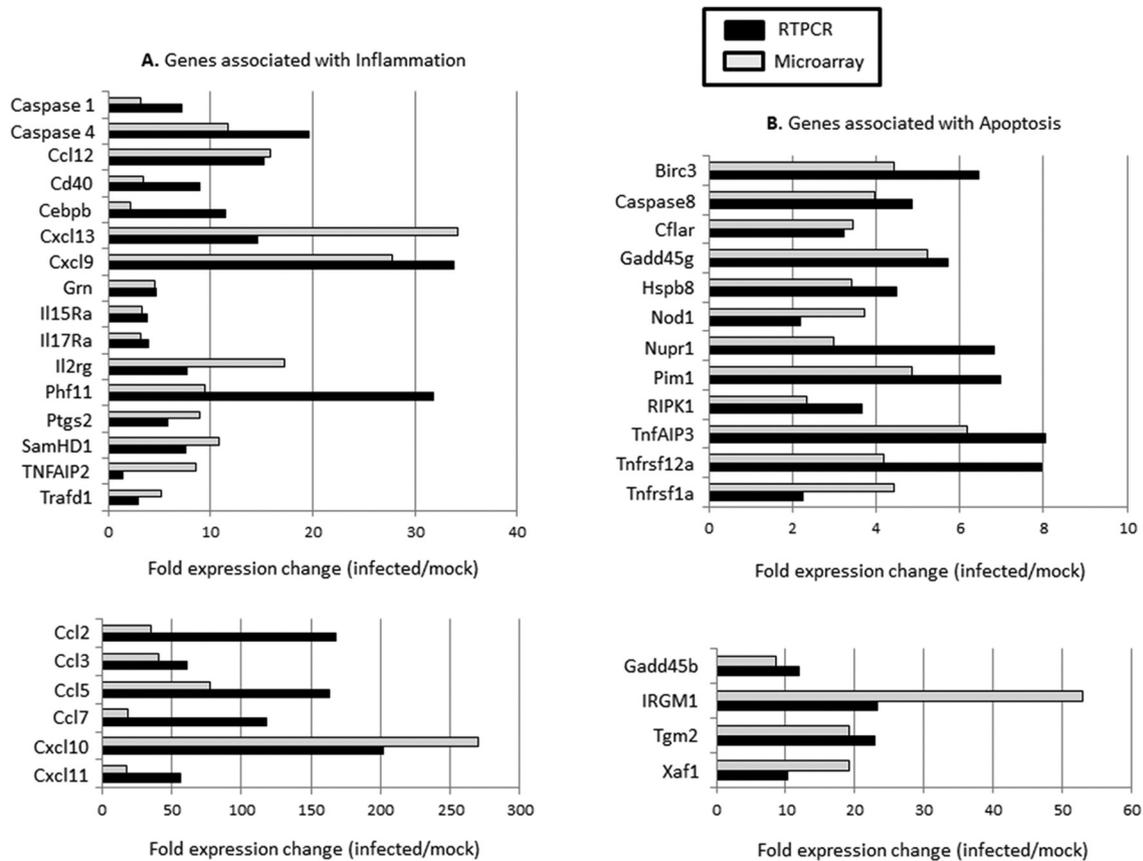
regulated following infection with JEV and that 57% (352/614) of genes that are up-regulated in the brain following infection with JEV are also up-regulated following infection with WNV (Fig. 1D; also, see Tables S1 and S2 in the supplemental material).

Results were also compared to gene expression changes in the brains of neonatal SW mice following i.c. inoculation with 1,000 PFU of T3D reovirus, an unrelated neurotropic virus (21). Brains were again harvested when animals displayed clinical symptoms of encephalitis (7 to 8 days postinfection). Five hundred eighty-two genes were up-regulated (criteria, a change of  $>2$ -fold and a  $P$  value of  $<0.001$ ) in the brain following infection with reovirus (Fig. 1A and C; also, see Table S3 in the supplemental material). Three hundred eighteen up-regulated genes were common to infections with WNV and reovirus, and 263 genes were common to infections with JEV and reovirus (Fig. 1D). Fifty-five percent (318/582) of genes that are up-regulated in the brain following infection with WNV are thus also up-regulated following infection with reovirus, and 43% (263/614) of the genes that are up-regulated in the brain following infection with JEV are also up-regulated following infection with reovirus. These results suggest that the number of genes induced following infection of the brain with viruses from the same family (two different flaviviruses) is more similar than that induced by viruses from different families (a flavivirus compared to a reovirus) and that reovirus and JEV are most different in the number of genes that are up-regulated following infection.

Two hundred sixteen up-regulated genes are common to infections with all three viruses (Fig. 1D). Thirty-five percent (216/614) of JEV-induced up-regulated genes are thus also up-regulated by WNV and reovirus, 37% (216/582) of WNV-induced up-regulated genes are also up-regulated by JEV and reovirus and 37% (216/582) of reovirus-induced up-regulated genes were also up-regulated following infection with JEV and WNV. Genes that are up-regulated in infection with all three viruses are mainly associated with interferon (IFN) signaling, the immune system, inflammation, and cell death/survival signaling (Table 1; also, see Table S4 in the supplemental material).



**FIG 1** Virus infection of the brain causes changes in cellular gene expression. Mice were infected with JEV (40 PFU), WNV (100 PFU), or reovirus (1,000 PFU) by i.c. inoculation. At 5 to 8 days postinfection, when mice were showing clinical manifestations of encephalitis, brains were harvested and RNA was analyzed using Affymetrix gene chips. (A) Volcano plots showing gene expression changes in the brain following infection with JEV, WNV, and reovirus. Each dot represents one gene on the Affymetrix gene chip. Genes with changes in expression greater than 2-fold ( $P < 0.001$ ) are indicated (larger dots). (B) Principal components analysis of microarray data. (C) Table showing the number of differentially expressed genes following infection with JEV, WNV, and reovirus. (D) Venn diagram showing the overlap of differentially expressed genes following virus infection of the brain. (E) Graph showing the number and fold change of up- and down-regulated genes ( $P < 0.001$ ) following virus infection.



**FIG 2** RT-PCR confirms differential gene expression identified by microarray. RT-PCR confirmation of select genes associated with inflammation (A) and cell death and survival signaling (B). The graphs show changes in expression (infected versus mock infected) following infection with WNV, as determined by RT-PCR and microarray analysis.

The magnitude of expression change in genes that were down-regulated in the brain following virus infection was much lower than that for up-regulated genes (Fig. 1E). Following WNV and reovirus infection, there was also a considerable decrease in the number of down-regulated compared to up-regulated differentially expressed genes (Fig. 1C). Five hundred two genes were found to be down-regulated (criteria, a change of  $>2$ -fold and a  $P$  value of  $<0.001$ ) in the brain following infection with JEV, 174 genes were down-regulated (criteria, a change of  $>2$ -fold and a  $P$  value of  $<0.001$ ) following infection with WNV, and 11 genes were down-regulated (criteria, a change of  $>2$ -fold and a  $P$  value of  $<0.001$ ) following infection with reovirus (see Tables S5 to S7 in the supplemental material). Additionally, there was less overall similarity between viruses in the number of down-regulated genes following infection. Eighty-six down-regulated genes were common to infections with JEV and WNV (Fig. 1C and D). Only 17% (86/502) of JEV-induced down-regulated genes and 49% (86/174) of WNV-induced down-regulated genes are thus down-regulated in infections with both flaviviruses (Fig. 1D; also, see Tables S5 and S6 in the supplemental material). Less than 1% of genes that were down-regulated by JEV or WNV were also down-regulated by reovirus (Fig. 1D), again indicating that infection with two different flaviviruses has a more similar effect on gene expression in the brain than infection with viruses from different families.

One down-regulated gene, encoding the protein known as sol-

ute carrier family 22 (organic anion transporter), member 8 (Slc22a8), was common to infections with all three viruses (Fig. 1D). Microarray data demonstrated that Slc22a8 was down-regulated  $-3.06$ -fold ( $P = 0.0005$ ) following infection with JEV,  $-4.05$ -fold ( $P = 0.0002$ ) following infection with WNV, and  $-2.66$ -fold ( $P = 8.16e-05$ ) following infection with reovirus (Tables S5 to S7).

**Confirmation of microarray data using RT-PCR.** Custom PCR arrays were used on additional virus-infected ( $n = 3$ ) and mock-infected ( $n = 3$ ) animals to confirm the gene expression changes of a selected number of genes that were up-regulated in infections with JEV, WNV, and reovirus. Twenty-two genes associated with inflammation were up-regulated in infections with JEV, WNV, and reovirus (Table 1). Reverse transcription-PCR (RT-PCR) arrays confirmed that 21 of these genes were up-regulated  $>2$ -fold in WNV-infected animals compared to mock-infected controls, and one gene (TNFAIP2) was up-regulated  $\geq 1.5$ -fold (Fig. 2). Similarly, RT-PCR arrays ( $>2$ -fold change) were used to confirm the up-regulation of 16 of the 17 genes associated with cell death and survival pathways (Table 1) that were found to be up-regulated in infections with JEV, WNV, and reovirus (Fig. 2). We previously confirmed the up-regulation of the additional gene (Daxx) following WNV infection of the brain using individual RT-PCRs and Western blot analysis (22, 23). Figure 2 demonstrates the similarity in the fold changes seen with

TABLE 2 Pathways identified in the brain by IPA of genes that are up-regulated following virus infection<sup>a</sup>

| General function | IPA pathway <sup>b</sup>                                | JEV                        |                    | WNV           |       | Reovirus      |       |
|------------------|---|----------------------------|--------------------|---------------|-------|---------------|-------|
|                  |   | –Log <i>P</i> <sup>c</sup> | Ratio <sup>d</sup> | –Log <i>P</i> | Ratio | –Log <i>P</i> | Ratio |
| IFN signaling    | IFN signaling   | 17.0                       | 0.47               | 16.4          | 0.44  | 10.4          | 0.33  |
|                  | Activation of IRF by cytosolic PRR                      | 14.8                       | 0.26               | 18.5          | 0.29  | 11.9          | 0.22  |
|                  | Role of PRR in recognition of bacteria and viruses      | 12.5                       | 0.21               | 10.7          | 0.19  | 11.5          | 0.19  |
|                  | Role of PKR in IFN induction and the antiviral response | 9.5                        | 0.28               | 12.7          | 0.33  | 7.73          | 0.24  |
| Immune response  | TREM 1 signaling  | 13.4                       | 0.25               | 11.9          | 0.24  | 13.0          | 0.25  |
|                  | Communication between innate and adaptive immune cells  | 12.0                       | 0.17               | 11.8          | 0.17  | 15.2          | 0.20  |
|                  | Antigen presentation                                    | 9.0                        | 0.28               | 8.29          | 0.25  | 16.9          | 0.16  |
| Apoptosis        | Death receptor signaling                                | 6.7                        | 0.19               | 14.0          | 0.28  | 8.2           | 0.20  |
|                  | Retinoic acid-mediated apoptotic signaling              | 7.0                        | 0.17               | 8.7           | 0.19  | 7.5           | 0.17  |
| Other            | IL-17RA signaling                                       | 8.0                        | 0.28               | 8.7           | 0.19  | 7.5           | 0.17  |

<sup>a</sup> Mice were infected with JEV, WNV, or reovirus. At 5 to 8 days postinfection brains were harvested and RNA was analyzed using Affymetrix microarrays. Ingenuity pathway analysis was performed on genes that were upregulated (change, >2.0-fold;  $P < 0.001$ ) following virus infection.

<sup>b</sup> IRF, interferon regulatory factor; PRR, pattern recognition receptor; PKR, protein kinase R; TREM 1, triggering receptor expressed on myeloid cells 1.

<sup>c</sup> IPA calculates the probability that genes belonging to a particular pathway are differentially regulated compared to other cellular genes.

<sup>d</sup> IPA calculates the number of differentially regulated genes divided by the total number of genes within a specific pathway (ratio).

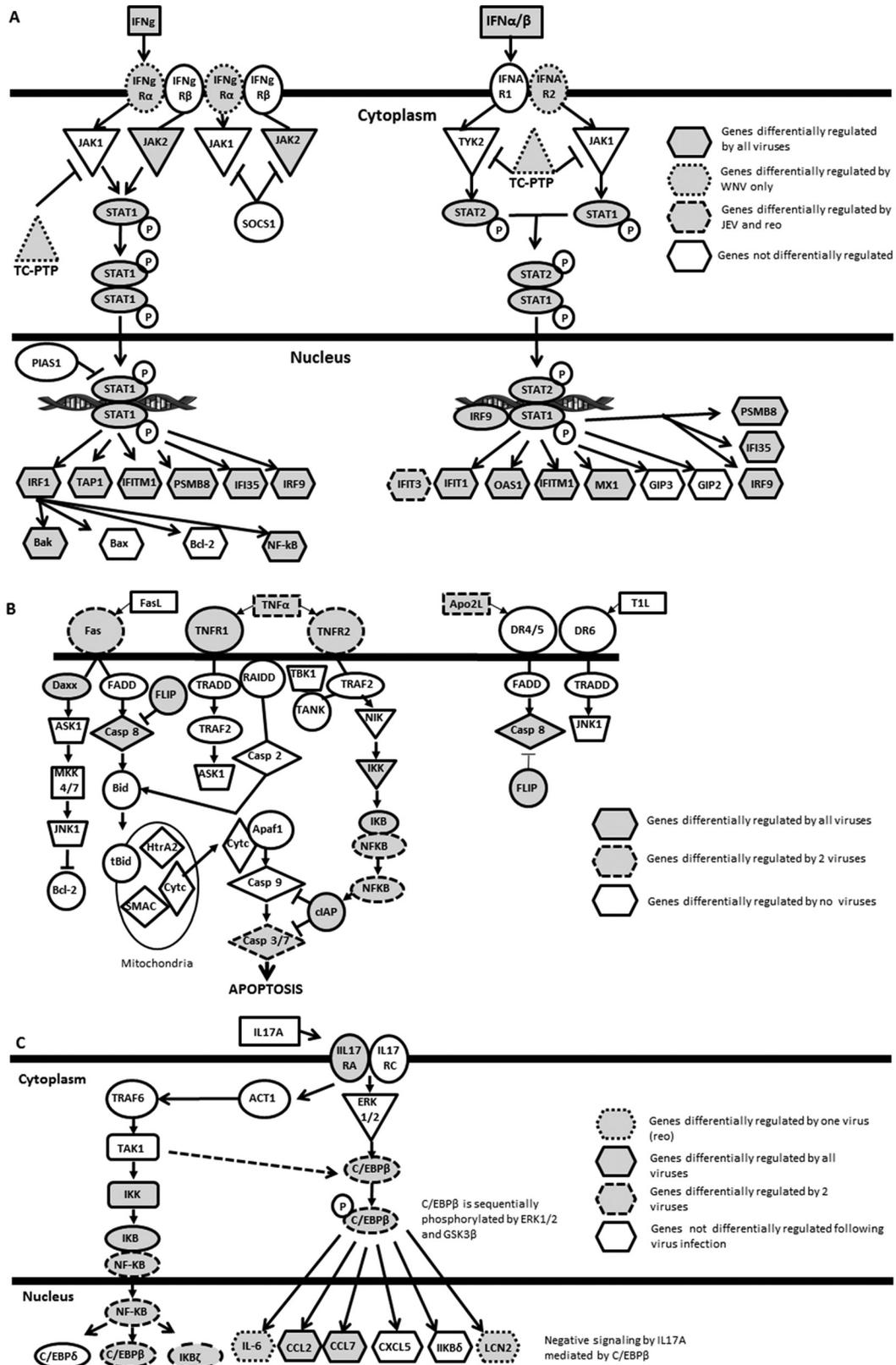
microarray and RT-PCR analysis for individual genes. RT-PCR was also used on RNA extracted from additional JEV-infected animals to confirm the up-regulation of all 22 genes associated with inflammation and 15 of the 17 genes that were associated with cell death and survival pathways that were up-regulated in infections with JEV, WNV, and reovirus (data not shown). The cell death and survival pathways that were not confirmed following RT-PCR of additional JEV- and mock-infected animals were TNFRsf1a and TNFRsf12a. These results confirm the validity of our microarray data. It is unclear whether the few anomalies were due to false positives/negatives in the different assays or due to animal-to-animal variation.

Ingenuity pathway analysis indicates that genes that are up-regulated following infection of the brain with different virus families are associated with innate immune responses.

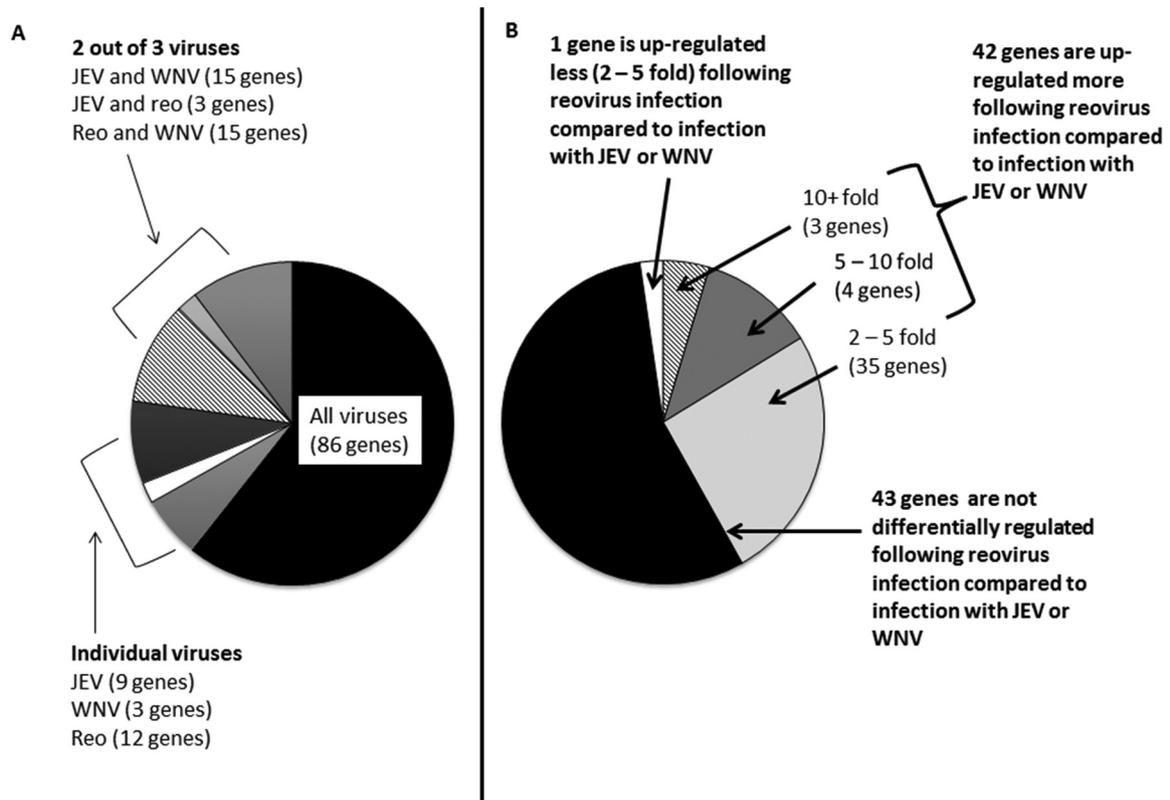
Ingenuity pathway analysis (IPA) was used to identify cellular signaling pathways that are associated with gene expression changes following viral infection. IPA calculates the probability that genes belonging to a particular pathway are differentially regulated compared to other cellular genes and the number of differentially regulated genes divided by the total number of genes within a specific pathway (ratio). Both these values can be used to identify cellular pathways that may be activated based on the pattern of differential gene expression. We performed IPA analysis on all genes that were differentially up-regulated with a fold change >2 ( $P < 0.001$ ). The top 10 pathways identified following infection with all three viruses were selected based on probability (–log probability  $\geq 6.7$ ) and ratio ( $\geq 0.16$ ) (Table 2). Consistent with results from the analysis of individual genes these pathways were associated with IFN and immune responses and apoptosis. The IFN- and immune response-associated pathway with the highest IPA ratios was “IFN Signaling” (Fig. 3A), and the apoptosis-associated pathway with the highest IPA ratios was “Death Receptor Signaling” (Fig. 3B). Interleukin 17 receptor A (IL-17RA) sig-

naling was also identified by IPA as being associated with infection by all three viruses (Fig. 3C).

Since IFN signaling had the highest IPA ratios following virus infection, we chose to further investigate this pathway following virus infection of the brain. The ingenuity pathway for IFN signaling includes only a select number of the hundreds of known IFN-regulated genes (Fig. 3A). To expand the number of IFN-regulated genes used for analysis, we used the interferome (24) database to identify virus-induced genes that have been previously been demonstrated to be differentially regulated in the brain by IFN. One hundred forty-three genes that were up-regulated following infection with JEV, WNV, or reovirus have previously been shown to be up-regulated in the brain following treatment with IFN (see Table S8 in the supplemental material). One hundred thirteen (79%) of these genes were up-regulated by JEV, 119 (83%) were up-regulated by WNV, and 116 (81%) were up-regulated by reovirus (see Table S8). Eighty-six (60%) of the 143 IFN-regulated genes were up-regulated following infection with all three viruses, suggesting there was more similarity in the up-regulation of IFN-regulated genes between different viral families than in total gene expression (see above) (Fig. 4A; also, see Table S8 in the supplemental material). Differences between virus families were not as apparent as those seen with overall gene expression. Thus, infections with reovirus and WNV had the same number of genes that were up-regulated in common compared to infections with JEV and WNV (Fig. 4A). The 86 genes up-regulated by all viruses included those with typical IFN-related functions, such as inflammation, immune-cell signaling, viral recognition, and antiviral responses (see Table S9 in the supplemental material). The interferon response induced by reovirus was more robust than that produced by JEV and WNV in that the expression of individual genes was increased more following reovirus infection than following infection with JEV or WNV. Forty-two of the 86 common genes were increased more than 2-fold following reovirus infec-



**FIG 3** Ingenuity pathway analysis identifies cellular signaling pathways that are associated with genes that are up-regulated in infections with JEV, WNV, and reovirus. IPA was performed on genes that were differentially up-regulated following virus infection (change, >2-fold;  $P < 0.001$ ). IPA identified genes involved in IFN signaling (A), death receptor signaling (B), and IL-17RA signaling (C) as being common to infections with JEV, WNV, and reovirus. The charts are adapted from IPA results. Shaded boxes represent genes that were differentially up-regulated following virus infection.



**FIG 4** Interferon-regulated genes are up-regulated in the brain following virus infection. The Interferome database was used to identify IFN-regulated genes that were up-regulated in the brain following infection with JEV, WNV, or reovirus. Pie charts were generated showing the number of IFN-regulated genes that were differentially up-regulated following infection with all 3 viruses, 2 of the 3 viruses, and individual viruses (A) and the number of IFN-regulated genes that were up-regulated to a higher degree following reovirus infection than following infection with JEV or WNV (B).

tion compared to infection with JEV or WNV (Fig. 4B). Genes whose expression was increased more than 5-fold following reovirus infection compared to infection with JEV or WNV included those encoding Z-DNA-binding protein 1 (ZBP1), Sp100, caspase 4, lymphocyte antigen 6 complex, locus A (Ly6A), interferon-induced protein 35 (Ifi35), and chemokine (C-X-C motif) ligand 11 (Cxcl11).

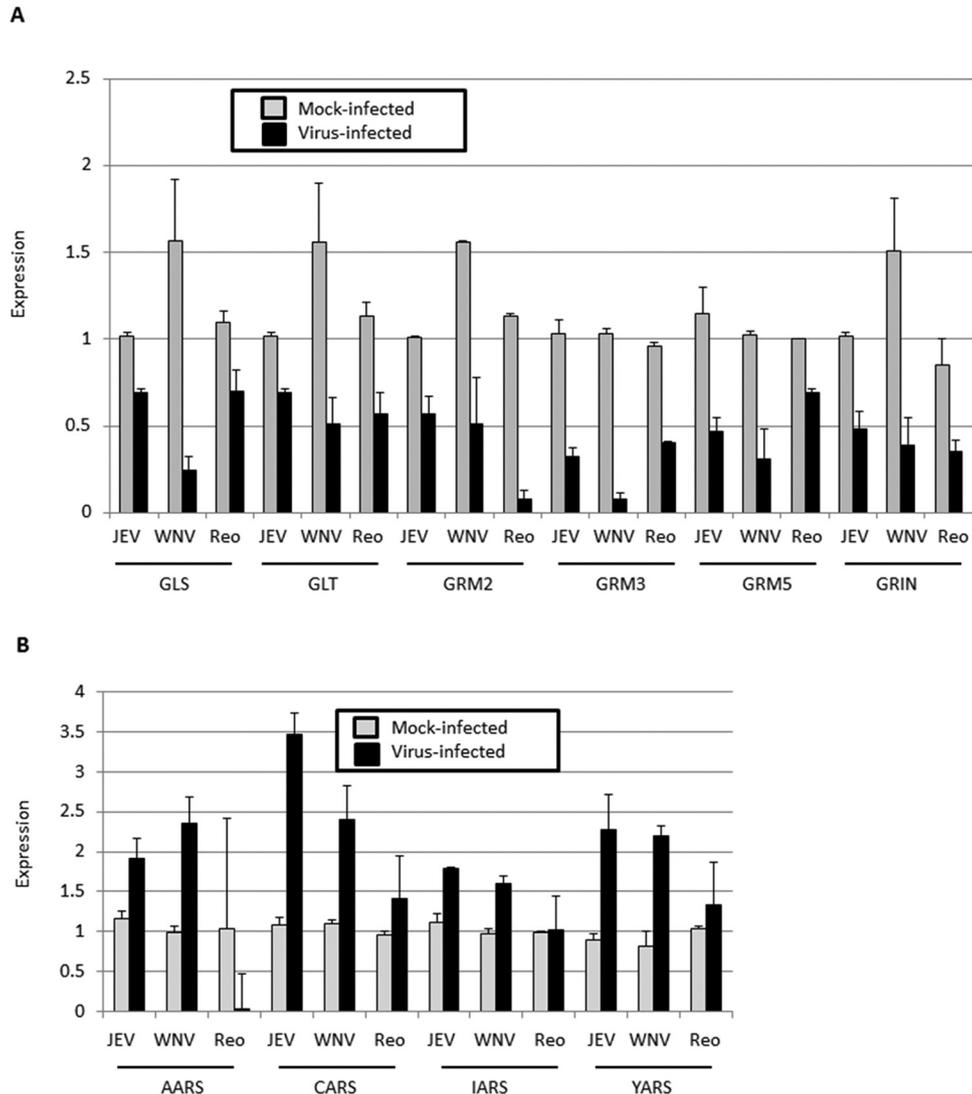
Ingenuity pathway analysis indicates that genes that are down-regulated following infection of the brain with different virus families are associated with glutamate signaling.

IPA was also used to identify pathways that were associated with genes that were down-regulated following virus infection. Glutamate signaling was the top pathway identified from genes that were down-regulated (change, >2-fold;  $P < 0.001$ ) following infection with JEV ( $-\log P = 3.9$ ; ratio =  $1.3 \times 10^{-1}$ ) and WNV ( $-\log P = 4.71$ ; ratio =  $8.7 \times 10^{-2}$ ). Analysis of individual genes indicated that 9 genes associated with glutamate signaling were down-regulated following JEV infection of the brain (change, >1.5-fold;  $P < 0.03$ ) as determined by microarray (Table 3). Specifically, the expression of multiple glutamate receptors and the

**TABLE 3** Genes associated with glutamate signaling are down-regulated following virus infection of the brain<sup>a</sup>

| Gene product | JEV         |          | WNV         |                       | Reovirus    |                       |
|--------------|-------------|----------|-------------|-----------------------|-------------|-----------------------|
|              | Fold change | <i>P</i> | Fold change | <i>P</i>              | Fold change | <i>P</i>              |
| GRIN         | -3.1        | 0.002    | -2.2        | 0.0002                | -1.2        | 0.0009                |
| GRM1         | -3.0        | 0.001    | -1.6        | 0.005                 | 1.2         | 0.05                  |
| GRM2         | -3.5        | 0.0007   | -2.1        | 0.03                  | -1.8        | 0.08                  |
| GRM3         | -5.9        | 0.0009   | -3.3        | $7.11 \times 10^{-5}$ | -1.2        | 0.001                 |
| GRM4         | -3.2        | 0.03     | -1.5        | 0.02                  | -1.3        | 0.006                 |
| GRM5         | -2.8        | 0.004    | -2.1        | 0.0007                | -1.1        | 0.03                  |
| SLC1A2       | -2.0        | 0.003    | -2.0        | 0.0007                | 1.2         | 0.08                  |
| GLS          | -1.9        | 0.0009   | -2.0        | 0.0004                | -2.0        | $2.44 \times 10^{-5}$ |
| SLC17A       | -2.2        | 0.0016   | -1.9        | 0.001                 | -1.5        | 0.005                 |

<sup>a</sup> Mice were infected with JEV, WNV, or reovirus. At 5 to 8 days postinfection, brains were harvested, and RNA was analyzed using Affymetrix microarrays. Data for expression of genes encoding glutamate receptor, ionotropic, *N*-methyl-D-aspartate (GRIN), glutamate receptor, metabotropic (GRM) 1 to 5, solute carrier family 1 (glial high-affinity glutamate transporter), member 2 (SLC1A2), glutaminase (GLS), and solute carrier family 17 (organic anion transporter), member 1 (SLC17A), are shown.



**FIG 5** RT-PCR confirmation of microarray data, showing that glutamate signaling and tRNA charging are associated with genes that are differentially expressed in the brain following virus infection. The graphs show RT-PCR confirmation of glutamate signaling genes that were shown by microarray analysis to be down-regulated following infection with JEV, WNV, and reovirus (A) and of tRNA synthetases that were shown by microarray analysis to be up-regulated following infection with JEV and WNV (B). RT-PCR was performed on virus-infected ( $n = 3$ ) and mock-infected ( $n = 3$ ) mice that were independent from those used for the microarray experiments. Columns represent average expression values. Error bars represent standard errors of the mean.

glutamate transporter GLT-1 was down-regulated in JEV-infected brains (Table 3). These genes were also identified by microarray as being down-regulated following WNV infection of the brain (change,  $>1.5$ -fold;  $P < 0.03$ ). Glutamate signaling was not identified by IPA from genes (change,  $>2$ -fold;  $P < 0.001$ ) that were down-regulated following reovirus infection. However, analysis of individual genes indicated that several genes associated with glutamate signaling were down-regulated following reovirus infection as determined by microarray, albeit with a lower degree of change (Table 3). Down-regulation of genes associated with glutamate signaling following JEV, WNV, and reovirus infections was confirmed by RT-PCR (Fig. 5A).

**Genes that are up-regulated following infection of the brain with flaviviruses but not an unrelated virus are associated with tRNA charging.** IPA was also used to identify pathways that were common to JEV and WNV infections but were not associated with

reovirus infection. The tRNA charging pathway was the only pathway that was identified ( $-\log P > 3.0$ ; ratio  $> 0.08$ ) from up-regulated genes (change,  $>2$ -fold;  $P < 0.001$ ) following JEV and WNV infections but was not identified ( $-\log P < 1.0$ ) following reovirus infection. Specifically, the up-regulation of 14 of the 20 tRNA synthetases was found to be common to infection of the brain with JEV or WNV (change,  $>1.4$ -fold;  $P < 0.003$ ) (Table 4). In contrast, none of these tRNA synthetases were up-regulated following infection with reovirus. The up-regulation of tRNA synthetases following JEV and WNV infection, but not following reovirus infection, was confirmed by RT-PCR (Fig. 5B).

## DISCUSSION

This report compares gene expression in the mouse brain following infection with the flaviviruses JEV and WNV. It also compares gene expression changes in the mouse brain following flavivirus

TABLE 4 tRNA synthetases are upregulated in infection of the brain with JEV or WNV<sup>a</sup>

| Gene product | Full name                    | JEV         |          | WNV         |          | Reovirus    |          |
|--------------|------------------------------|-------------|----------|-------------|----------|-------------|----------|
|              |                              | Fold change | P        | Fold change | P        | Fold change | P        |
| AARS         | Alanyl-tRNA synthetase       | 2.23        | 3.7e-06  | 2.15        | 4.97e-06 | 1.07        | 0.13     |
| CARS         | Cyseinyl-tRNA synthetase     | 4.53        | 9.25e-05 | 3.64        | 7.07e-06 | 2.38        | 0.13     |
| GARS         | Glycyl-tRNA synthetase       | 1.95        | 0.0001   | 1.92        | 7.72e-07 | 1.08        | 0.03     |
| IARS         | Isoleucyl-tRNA synthetase    | 2.09        | 9.9e-06  | 2.2         | 0.0005   | -1.04       | 0.09     |
| LARS         | Leucyl-tRNA synthetase       | 2.84        | 0.0005   | 2.02        | 0.0002   | 1.08        | 0.004    |
| MARS         | Methionyl-tRNA synthetase    | 1.59        | 0.003    | 1.70        | 0.001    | 1.11        | 0.007    |
| NARS         | Asparaginyl-tRNA synthetase  | 2.03        | 0.0001   | 1.44        | 1.27e-05 | 1.05        | 0.98     |
| RARS         | Arginyl-tRNA synthetase      | 2.91        | 2.6e-05  | 2.10        | 0.0001   | 1.21        | 0.01     |
| SARS         | Seryl-tRNA synthetase        | 1.73        | 0.0005   | 1.53        | 0.001    | 1.01        | 0.26     |
| TARS         | Threonyl-tRNA synthetase     | 2.51        | 0.0004   | 2.43        | 0.002    | 1.28        | 0.003    |
| VARS         | Valyl-tRNA synthetase        | 1.9         | 0.0005   | 1.8         | 0.001    | 1.17        | 0.34     |
| WARS         | Tryptophanyl-tRNA synthetase | 2.11        | 0.0007   | 1.94        | 0.0008   | 1.31        | 4.97e-05 |
| YARS         | Tyrosyl-tRNA synthetase      | 2.07        | 0.0001   | 2.02        | 4.11e-05 | -1.05       | 0.47     |
| EPRS         | Glutaminyl-tRNA synthetase   | 2.23        | 4.5e-05  | 1.85        | 0.0002   | -1.08       | 0.53     |

<sup>a</sup> Mice were infected with JEV, WNV, or reovirus. At 5 to 8 days postinfection, brains were harvested, and RNA was analyzed using Affymetrix microarrays.

infection to those induced following infection with T3D reovirus, an unrelated neurotropic virus. To our knowledge, this is the first report to directly compare global gene expression changes between different genera and different families of viruses.

**Upregulated genes.** Around 600 genes were found to be upregulated (criteria, a change of >2-fold and a *P* value of <0.001) in the brain following viral infection, regardless of the virus used (JEV, WNV, or reovirus). The number of genes that were upregulated in infections with different flaviviruses was higher than the number of genes that were upregulated in infections with viruses from different families. Two hundred sixteen upregulated genes were common to infections with all three viruses and included genes associated with IFN signaling, the immune system, inflammation, and cell death/survival signaling. Consistent with these results, Ingenuity pathway analysis identified IFN and immune responses, apoptosis, and IL-17RA signaling as being associated with infection of all 3 viruses (-Log *P* ≥ 6.7, ratio ≥ 0.16). Pathway and individual gene analysis also identified tRNA charging as being associated with infection of the brain with JEV and WNV but not with reovirus.

**(i) IFN signaling.** The activation of IFN and immune response pathways following viral infection of cell and tissues (including the CNS) has been seen following infection with a variety of viruses, and it is well known that IFN signaling plays a protective role following virus infection (25). In addition to the direct antiviral effects of IFN- $\alpha/\beta$ , these cytokines also modulate the immune system by activating effector cell function and promoting the development of the acquired immune response. Expression of ISGs has been reported in the CNS after infection with many viruses, including JEV (17, 18), WNV (13, 26), and reovirus (21). Further, increases in viral load within the CNS and increased susceptibility to viral infection are seen in mice deficient for the type 1 IFN receptor (IFNAR) or other components of the host interferon response following infection with many viruses.

In this study, we compared the IFN responses generated by different viruses. We used the interferome database to identify 143 IFN-associated genes that were upregulated in the brain following infection with JEV, WNV, or reovirus and that were previously

shown to be up-regulated in the CNS by IFN. Compared to overall gene expression profiles, there was greater similarity in the number of genes that were up-regulated in infections with all three viruses. Thus, 86 (60%) of the 143 genes were up-regulated following infection with all three viruses. The differences between virus families were also not as apparent as those seen with overall gene expression (see above), and infections with reovirus and WNV had the same number of genes that were up-regulated in common compared to infections with JEV and WNV. The 86 genes up-regulated by all viruses included those with typical IFN-related functions, such as inflammation, immune cell signaling, viral recognition, and antiviral responses. The interferon response induced by reovirus was more robust than that produced by JEV or WNV in that the expression of individual genes was increased following reovirus infection compared to the increase seen following infection with JEV or WNV. This is consistent with previous studies documenting inhibition of IFN signaling following infection with WNV (27–33), JEV (34, 35), and other members of the *Flaviviridae*. Notable genes that were up-regulated more than 5-fold following reovirus infection compared to infection with JEV or WNV included those encoding ZBP1, Sp100, caspase 4, lymphocyte antigen 6 complex, locus A (Ly6A), interferon-induced protein 35 (Ifi35), and chemokine (C-X-C motif) ligand 11 (Cxcl11). Although the functions of these proteins in viral pathogenesis remains unclear, possible reasons why Zbp1 is up-regulated to a higher degree in reovirus infect might include the fact that Zbp1 can recognize and stabilize double-stranded RNA (dsRNA) (36), which might be beneficial to the host in limiting reovirus replication. In addition, Cxcl11, which is chemotactic for interleukin-activated T cells, may be specifically down-regulated following WNV infection to inhibit T cell infiltration, which is known to be an important factor in WNV clearance (37).

**(ii) Apoptosis signaling.** Apoptosis signaling was associated with genes that were up-regulated following infection with all three viruses by analysis of individual genes and by pathway analysis. This is consistent with the known association of viral CNS pathogenesis with apoptotic cell death and caspase 3 activation in *in vivo* models (38). *In vivo* and *in vitro* studies have pointed to

various mechanisms that trigger caspase 3 activation and apoptosis following viral infection, including death receptor-mediated apoptosis and activation of the initiator caspase, caspase 8 (23, 39, 40), as well as mitochondrial apoptotic signaling and the activation of caspase 9 (23, 40, 41). In this study, we found that genes associated with cell death and apoptosis that were up-regulated in JEV, WNV, and reovirus infection included genes associated with death receptor signaling (those encoding TNFRSF1a, caspase 8, and Daxx), mitochondrial apoptotic signaling (that encoding Nod1), and those encoding other proapoptotic proteins with less defined roles, such as transglutaminase 2 (Tgm2), Growth Arrest and DNA-Damage-Inducible gamma (gadd45g) and beta (Gadd45b), and tumor necrosis factor-like weak inducer of apoptosis (TWEAK).

Several genes with antiapoptotic or prosurvival functions were identified as being up-regulated in JEV, WNV, and reovirus infections of the brain, including those encoding tumor necrosis factor alpha (TNF- $\alpha$ )-induced protein 3 (TNFAIP3), Pim1, CASP8 and FADD-like apoptosis regulator (cFLAR), baculoviral IAP repeat containing 3 (BIRC3), and nuclear protein, transcriptional regulator, 1 (Nupr1).

**(iii) IL-17RA signaling.** IL-17 is a proinflammatory cytokine that induces the expression of many mediators of inflammation and that has been associated with various chronic inflammatory conditions, including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and cancer. IL-17 responses have also been associated with bacterial infections (42). Our observation that genes that are up-regulated following in the mouse brain in infections with JEV, WNV, and reovirus are associated with IL-17RA signaling suggest a role for IL-17 in virus-induced pathogenesis within the CNS. The presence of a homolog of IL-17 in the herpesvirus saimiri (HVS) genome (43, 44) has previously pointed to the importance of IL-17 in virus infections. Indeed, vaccinia virus expressing IL-17 is more virulent in mice and elicits decreases in NK cell toxicity compared to its parental control virus (45), indicating that IL-17 modulates the immune response during vaccinia virus infection and emphasizing the importance of its role in NK cell activation. IL-17 is also expressed in the corneas of patients with herpes stromal keratitis (46) and in HSV-1-infected mouse corneal fibroblasts (47). Ligand-receptor interaction significantly influences the host response to HSV-1 infection by producing the proinflammatory mediators IL-6 and MIP-2 and determining neutrophil influx (47).

**(iv) tRNA charging.** We report an increase in the expression of multiple tRNA synthetases in the brain following flavivirus infection but not following infection with reovirus. An increase in the protein expression of tRNA synthetases was also seen following WNV infection of Vero cells (15). This suggests that the up-regulation of tRNA synthetases may be required for flavivirus propagation and is consistent with the fact that flaviviruses do not shut off host protein cell translation and must compete with the cellular translational machinery for limiting factors.

**Down-regulated genes.** The magnitude of expression change in genes that were down-regulated in the brain following virus infection was much lower than that for up-regulated genes, and there was a considerable decrease in the number of down-regulated (fold change  $>2$ ,  $P < 0.001$ ), compared to up-regulated, differentially expressed genes following WNV and reovirus infection. Additionally, there was less overall similarity between viruses in the number of down-regulated genes following infection. Com-

parison of down-regulated genes again indicated that infection with 2 different flaviviruses has a more similar effect on gene expression in the brain than viruses from different families. Only one down-regulated gene, encoding solute carrier family 22 (organic anion transporter), member 8 (Slc22a8), was common to infections with all three viruses. Solute carrier family 22, member 8, plays an important role in the excretion/detoxification of endogenous and exogenous organic anions, especially from the brain and kidney (48).

Pathway (Ingenuity) and individual gene analysis along with RT-PCR identified glutamate signaling as being associated with genes that were down-regulated following infection with all three viruses. Specifically, the expression of multiple glutamate receptors and the glutamate transporter GLT-1 was down-regulated in virus-infected brains. Glutamate is the primary excitatory neurotransmitter in the neurological system. Previous work demonstrated that an excess of glutamate at the synaptic cleft may lead to apoptosis of neurons through glutamate excitotoxicity and may contribute to pathology in many neurological conditions, including viral infections (49–53). Humans and hamsters with WNV-induced acute flaccid paralysis (AFP) also have decreased expression of GLT-1 despite greater numbers of activated astrocytes, leading to the suggestion that local immune activation within the spinal cord causes a failure of astrocyte glutamate reuptake, rising extracellular glutamate levels, and excitotoxic injury. Further, glutamate signaling pathways were also identified as having predicted relevance for WNV infection in the thalami and cerebrums of horses experimentally infected with WNV (54). In that study, horses that had not been vaccinated with a modified live attenuated yellow fever (YF) chimera vaccine for protection against WNV (Prevenile; Intervet-Schering-Plough) demonstrated gene expression changes consistent with glutamate excitotoxicity following exposure to WNV compared to both the vaccinated and normal (nonexposed) control horses (54). This again suggests that infection with WNV may lead to a down regulation of glutamate receptors, leading to an increase in glutamate levels and pathology associated with glutamate excitotoxicity. JEV also elicits the extracellular accumulation of glutamate following infection of microglia (55). Our finding that genes associated with glutamate signaling are down-regulated following infection with JEV, WNV, and reovirus suggests that glutamate excitotoxicity is an important broad-spectrum mechanism of virus-induced CNS disease.

## MATERIALS AND METHODS

**Mouse infections.** Stocks of WNV (clone derived 382-99 NY99 strain) and JEV (P3 strain) were prepared as previously described (56, 57). Adult Swiss Webster mice were infected with 100 PFU WNV or 40 PFU JEV by intracerebral (i.c.) inoculation. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and were performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility.

**RNA purification.** In order to generate the most consistent data for our microarray studies particular care was taken to standardize infections for RNA extractions. Adult male SW mice were infected with virus by i.c. inoculation. At 5 to 6 days postinfection (dpi), when infected mice were showing neurologic symptoms, mice were sacrificed, and brains from mock- and virus-infected mice were immediately placed in RNAlater and stored (short term) at  $-20^{\circ}\text{C}$ . Approximately 160 mg of brain tissue (about half of the brain after the cerebellum was removed) was homogenized in 1 ml of Qiazol (RNeasy lipid tissue minikit; catalog no. 74804; Qiagen) for at least 20 strokes until completely emulsified. The emulsion

was transferred to a clean 1.5-ml tube and set aside for at least 5 min before addition of 200  $\mu$ l chloroform. The mixture was shaken for 15 s and set aside for 5 min before centrifugation at 12,000  $\times$  g for 15 min at 4°C. The upper aqueous phase was then carefully removed and transferred to a new tube. One volume of 70% ethanol (prepared with diethyl pyrocarbonate [DEPC] water) was then added, and the solution was mixed before being transferred to an RNeasy column. RNA was purified following the manufacturer's specifications, and 0.5 to 1  $\mu$ l of RNasin was added to the sample, which was then stored at  $-80^{\circ}\text{C}$ .

**Microarray analysis.** Affymetrix 1.0 ST mouse whole-genome chips (Santa Clara, CA) were used for microarray analysis of RNA extracted from virus-infected and mock-infected brains. For each virus, RNA from 3 virus-infected mice and 3 mock-infected mice was loaded onto individual microarrays according to the manufacturer's specifications. All microarray experiments and initial analysis were performed at the microarray core facility (University of Colorado, Denver, CO). Analysis was performed using R statistical computation software and packages from Bioconductor open-source software for bioinformatics (58). Prior to statistical analysis, two preprocessing steps involving normalization and gene filtering were performed. Raw data from array scans were processed using the robust multichip average (RMA) normalization method to subtract a background value that is based on modeling the perfect match (PM) signal intensities as a convolution of an exponential distribution of signal and a normal distribution of nonspecific signal while ignoring the mismatch (MM) signal (59). After normalization, data were filtered using two criteria: (i) Affymetrix mRNA detection calls were used to exclude all probe sets with an "absent" call in all samples, and (ii) transcripts that demonstrated little variation across all arrays were removed. This was performed by comparing the variance of the log intensities for each gene with the median of all variance for the entire array. Genes not significantly more variable than the median were filtered out.

Principal components analysis (PCA) was used to compare gene expression data. Principal components analysis is an exploratory multivariate statistical technique for simplifying complex data sets. The goal of PCA is to reduce the dimensionality of the data by finding new variables, termed principal components. Each principal component is a linear combination of the original variables. Principal components analysis has been used in a wide range of biomedical problems, including the analysis of microarray data (60).

**Ingenuity pathway analysis.** Differentially regulated genes were analyzed using the Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, CA). The Ingenuity Pathways Analysis knowledge base is a curated database based on scientific evidence from thousands of journal articles, textbooks, and other data sources. IPA software was used to define which well-characterized cell signaling pathways are most relevant during viral encephalitis. The significance (*P* values) of the association between the data set and the canonical pathway was measured by comparing the number of genes that are differentially regulated during reovirus encephalitis that participate in a pathway relative to the total number of genes in all pathway annotations stored in the Ingenuity knowledge base. Fisher's exact test was used to calculate a *P* value reflecting the probability that the association between the genes in the data and the canonical pathway is explained by chance only.

**RT-PCR.** cDNA was prepared from purified RNA by reverse transcribing 1.0  $\mu$ g of each RNA sample using a SABiosciences first-strand kit (C-03; SABiosciences, Frederick, MD). The 20- $\mu$ l final volume of cDNA was diluted with 1,275  $\mu$ l of water and added to 1,275  $\mu$ l of 2 $\times$  SABiosciences RT2 SYBR green master mix (PA-010). A 25- $\mu$ l portion of this mixture was dispensed into each well of a SABiosciences RT2 profile IFN- $\alpha/\beta$  signaling (PAMM-14) or cytokine (PAMM-11) PCR array. RT-PCR was carried out on a Bio-Rad Opticon2 machine (Bio-Rad, Hercules, CA). Statistical analysis was performed using GraphPad software (Instat).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00902-14/-/DCSupplemental>.

Table S1, XLSX file, 0.1 MB.

Table S2, XLSX file, 0.1 MB.

Table S3, XLS file, 0.1 MB.

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Table S9, DOCX file, 0.1 MB.

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