Differentiation of Neurons Restricts Arbovirus Replication and Increases Expression of the Alpha Isoform of IRF-7

Kimberly L. W. Schultz, Patty S. Vernon, and Diane E. Griffin

W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205

Running title: Antiviral priming of differentiated neurons

1Present address: Scripps Mercy Hospital, San Diego, CA

Key words: interferon, neuronal maturation, IRF-3, IRF-7, Sindbis virus, Venezuelan equine encephalitis virus, La Crosse virus, arbovirus

#Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Medicine, 615 N. Wolfe St., Rm E5132, Baltimore, MD 21205. Phone: 410-955-3459. Fax: 410-955-0105. Email: dgriffin@jhsph.edu

Abstract word count – 222
Importance word count - 137
Text word count – 5,732
ABSTRACT

Susceptibility to alphavirus infection is age-dependent and host maturation is associated with decreased virus replication and less severe encephalitis. To identify factors associated with maturation-dependent restriction of virus replication, we studied AP-7 rat olfactory bulb neuronal cells that can be differentiated in vitro. Differentiation was associated with a 150- to 1000-fold decrease in replication of Sindbis and Venezuelan equine encephalitis alphaviruses, as well as La Crosse bunyavirus. Differentiation delayed synthesis of SINV RNA and protein, but did not alter the susceptibility of neurons to infection or virion maturation. Additionally, differentiation slowed virus-induced translation arrest and death of infected cells. Differentiation of uninfected AP-7 neurons was associated with changes in expression of antiviral genes. Expression of key transcription factors was increased, including interferon regulatory factor-3 and -7 (IRF-3 and IRF-7) and STAT-1, suggesting that neuronal maturation may enhance the capacity for antiviral signaling upon infection. IRF-7 produced by undifferentiated AP-7 neurons was exclusively the short dominant-negative γ isoform while that produced by differentiated neurons was the full-length α isoform. A similar switch in IRF-7 isoforms also occurred in the brains of maturing C57BL/6J mice. Silencing of IRF expression did not improve virus multiplication in differentiated neurons. Therefore, neuronal differentiation is associated with up regulation of transcription factors that activate antiviral signaling, but this alone does not account for maturation-dependent restriction of virus replication.

IMPORTANCE

Viral encephalomyelitis is an important cause of age-dependent morbidity and mortality. Because mature neurons are not readily regenerated, recovery from encephalitis
suggests that mature neurons utilize unique antiviral mechanisms to block infection
and/or clear virus. To identify maturational changes in neurons that may improve
outcome, we compared immature and mature cultured neurons for susceptibility to three
encephalitic arboviruses and found that replication of Old and New World alphaviruses
and a bunyavirus were reduced in mature compared to immature neurons. Neuronal
maturation was associated with increased baseline expression of interferon regulatory
factors -3 and -7 mRNAs, and production of distinct isoforms of interferon regulatory
factor-7 protein. Overall, our studies identify maturational changes in neurons that likely
contribute to assembly of immunoregulatory factors prior to infection, a more rapid
antiviral response, increased resistance to virus infection, and improved survival.

INTRODUCTION

Development of age-dependent resistance to fatal disease is a characteristic of many
virus infections of the central nervous system (CNS) (1-9). We have used Sindbis virus
(SINV), the prototype alphavirus in the family Togaviridae, as a model system to
understand maturation-mediated restriction of virus multiplication in neurons. SINV
preferentially infects neurons and causes age-dependent encephalomyelitis in mice.
Young animals are highly susceptible to SINV infection and succumb within 3 to 4 days.
In contrast, adult animals infected with the same strain of SINV restrict virus replication
in the CNS, are able to clear virus, and recover from infection (4). Age-dependent
restriction of virus replication is not due to the maturation of the immune system, but
rather to a decreased susceptibility of maturing neurons to infection (10, 11). The
molecular basis for this maturation-dependent restriction of virus replication is unclear,
but has previously been attributed to decreased expression of viral receptors,
proapoptotic molecules, and inflammatory response genes and to increased expression
of fractalkine and interferon (IFN)-inducible genes (12, 13). Although changes in neuronal receptor expression may contribute to age-dependent susceptibility to SINV (14), the reduced production of virus by mature neurons compared to immature neurons suggests a post-entry restriction of replication (15). Because neurons are terminally differentiated essential cells, for host recovery, neural function must be preserved during viral clearance. Therefore, neurons are uniquely important cells for the study of antiviral responses (16, 17).

Type I IFN signaling constitutes the first line of host defense against many virus infections (18, 19). IFN treatment of cells prior to SINV infection blocks replication (20) and mice deficient in IFN-α/β signaling are highly susceptible to infection (21, 22). IFN signaling is vital for early control of SINV infection in vivo (21, 23, 24) and susceptibility of Venezuelan equine encephalitis virus (VEEV) to IFN is a major determinant of attenuation and age-dependent susceptibility (25, 26). Similarly, deletion of the IFN antagonist NSs from La Crosse virus (LACV) decreases virulence in mammals and allows neuronal production of type I IFN during infection (27-29). These studies suggest a potential role for IFN signaling in maturation-dependent neuronal restriction of neuronotropic virus replication. Neurons can induce IFN signaling upon infection (28, 30-33) and IFN expression is increased during maturation of cultured neurons (34).

However, autocrine IFN signaling has not been implicated in maturation-dependent control of virus replication (33).

Canonically, the innate response to infection with RNA viruses is dependent on detection of viral RNA through surface interaction with Toll-like receptors or cytoplasmic interaction with RNA helicases such as RIG-I or MDA-5 (35, 36). These signaling pathways activate constitutively expressed IFN regulatory factors (IRFs) leading to expression of type I IFN and IFN-stimulated genes (ISGs). Thus, small amounts of IFN can stimulate increased ISG expression and facilitate an enhanced response to
subsequent virus infection, a phenomenon known as IFN-priming (37). Likewise, increased expression of proteins in the IFN induction pathway, such as RIG-I, increases resistance to virus infection (36, 38, 39).

Pathogen detection pathways converge on activation of IRF-3 and IRF-7, critical transcription factors for Type I IFN gene expression. IRF-3 is ubiquitously expressed and is the primary regulator of IFN β expression (40, 41). IRF-7 expression is regulated in a cell-type dependent manner and is required for maximal Type I IFN α gene expression (42, 43). Loss of IRF-3 and/or IRF-7 greatly impairs the immune response to infections in a virus and cell-type specific manner (44-47). IRFs can stimulate ISG expression in response to virus infection independent of IFN suggesting that increased IRF expression could also have a direct protective effect (48). For example, IRF-3 mediates innate immune signaling in neurons during western equine encephalitis virus and St. Louis encephalitis virus infection independent of IFN signaling (49).

To investigate the molecular mechanisms underlying maturation-dependent restriction of neuronotropic virus replication we have used cultured AP-7 rat olfactory sensory neurons immortalized by a temperature-sensitive SV40 T antigen (50). Similar to the CSM14.1 nigral neuron system (15), these neurons can be differentiated in vitro and thus allow comparison of virus replication and host antiviral responses in undifferentiated cycling (cAP-7) and differentiated non-dividing (dAP-7) neurons. Rats are susceptible to SINV-induced encephalomyelitis (51) and cultured immature and mature primary rat dorsal root ganglion neurons show maturation-dependent restriction of SINV replication (52).

dAP-7 neurons had an intrinsic ability to restrict multiplication of three different neuronotropic viruses compared to cAP-7 neurons. To determine the molecular mechanisms contributing to the intrinsic resistance of maturing neurons to neuronotropic viruses, we assessed the expression of cellular factors that induce and amplify the
cellular antiviral response. We found that neural maturation was associated with increased expression of immunoregulatory transcription factors Irf-3 and Irf-7 and further induction upon infection.

MATERIALS AND METHODS

Cell culture. Rat AP-7 *odora* neurons, an olfactory-derived cell line immortalized with a temperature-sensitive simian virus 40 T antigen (a gift from Dale Hunter; Tufts University, Boston MA) (50), were grown at 33°C in 7% CO₂ in DMEM/10% FBS supplemented with 100 U penicillin/mL, 100 µg streptomycin/mL and 2mM glutamine. At about 25% confluence, cells were differentiated for 5-7 days by shifting to 39°C and 5% CO₂ in DMEM/10% FBS supplemented with 1 µg/mL insulin, 20µM dopamine, 100 µM ascorbic acid, penicillin, streptomycin and glutamine. CSM14.1 neurons were grown in DMEM/10% FBS with penicillin, streptomycin and glutamine at 31°C in 5% CO₂. For differentiation, CSM14.1 cells were shifted to the nonpermissive culture conditions of DMEM/1% FBS with penicillin, streptomycin and glutamine at 39°C in 5% CO₂ for at least 3 weeks as described previously (15). BHK-21 cells were grown at 37°C in 5% CO₂ in DMEM/10% FBS with penicillin, streptomycin and glutamine. Cells were monitored for mycoplasma with the MycoAlert Mycoplasma detection kit (Lonza Rockland Inc, Rockland ME) and determined to be free of contamination.

Viruses and infection of cells. SINV strain TE (53), VEEV strain TC-83, (a gift from Ilya Frolov, University of Alabama, Birmingham AL), and La Crosse virus strain La Crosse/original (54) (a gift from Andrew Pekosz, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD) were used. Virus stocks were grown in and titers were determined by plaque formation on BHK-21 cells. AP-7 or CSM14.1 monolayers were infected with viruses at the indicated MOI in DMEM/1% FBS for 1 h, washed with PBS.
(pH 6.2) and medium was replaced. To quantify one-step virus production, neuronal monolayers were inoculated as described for each experiment. At each timepoint, 100μL of supernatant fluid was collected from each of 3 wells. Infectious virus was quantified by plaque assay on BHK-21 cells. Percent infected cells was determined by immunofluorescence microscopy by using monoclonal antibodies to E2 of either SINV (MAB209; (55)) or VEEV (1A3B7; (56)) with Alexa Flor 594-conjugated anti-mouse IgG (Life Technologies). The number of infected cells was compared to the total number of cells as visualized by DAPI-stained nuclei (ProLong Gold, Life Technologies).

Transmission Electron Microscopy. Samples were fixed in 2.5% glutaraldehyde, 3mM CaCl₂, 1% sucrose, in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h at room temperature. After buffer rinse, samples were postfixed in 1% osmium tetroxide in buffer (1 h) on ice in the dark. Following a dH₂O rinse, plates were stained with 2% aqueous uranyl acetate (0.22 μm filtered, 1 h, dark), dehydrated in a graded series of ethanol and embedded in Eponate 12 (Ted Pella) resin. Samples were polymerized at 60°C overnight.

Thin sections, 60 to 90 nm, were cut with a diamond knife on the Reichert-Jung Ultracut E ultramicrotome and picked up with naked 200 mesh copper grids. Grids were stained with 2% uranyl acetate in 50% methanol and observed with a Hitachi 7600 TEM at 80 kV. Images were captured with an AMT CCD (1K x 1K) camera.

Antisera and immunoblot analysis. For immunoblot analysis, neuronal monolayers were washed with cold 1X PBS (pH 6.2), lysed with cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1% SDS, 1% NP40, 0.5% NaDeoxycholate, 1 mM EDTA), incubated on ice for 30 min and cleared by centrifugation. Brain from 3-day or 6-week old C57BL/6J mice were homogenized in 1X PBS and cleared by centrifugation. 10 μg of total protein was
separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (BioRad). Immunodetection was conducted with the following antisera diluted 1:1000 unless otherwise indicated: monoclonal α-E2 (209; 1:2000) (55), polyclonal α-nsP3 (57), polyclonal α-IRF-7 (Sigma), polyclonal α-IRF-3 (Santa Cruz Biotechnology), polyclonal α-phospho-STAT-1(Y701) (Cell Signaling), and polyclonal α-STAT-1 (Cell Signaling) and monoclonal α-β actin (1:5000; Millipore). The membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse immunoglobulin-G (GE Healthcare) and developed using ECL Prime Western Blotting Reagents (GE Healthcare).

Measurement of RNA levels. Total cellular RNA was isolated using QIAGen RNeasy or RNeasy Plus minikits per the manufacturer’s directions. SINV positive strand RNA was quantified as described previously (57). Briefly, cDNA from total cellular RNA was transcribed using the ABI High Efficiency Reverse Transcription kit with a SINV-specific primer SINV9899R 5′ AGCATTGGCCGACCTAACGCAGCAC 3′. qPCR was performed on an ABI 7500 thermocycler with ABI qPCR mastermix using primers SINVE2F 5′ TGGGACGAAGCGGACGATAA 3′ and SINVE2R 5′ CTGCTCCGCTTTGGTCGTAT 3′, and Taqman probe 5′[FAM] CGCATACAGACTTCCGCCCAGT [TAMRA] 3′. Viral RNA was quantified by comparison to a standard of SINV genomic RNA.

Equal microgram amounts of RNA isolated from AP-7 neurons from 3 independent experiments were pooled and analyzed using the rat antiviral response qPCR array according to the manufacturer’s directions (PARN-122; SAbiosciences). cDNA was synthesized from cellular RNA using random primers and the ABI High Efficiency Reverse Transcription kit. ddCT qPCR was conducted in an ABI 7500 thermocycler with ABI qPCR mastermix. The following PrimeTime Standard qPCR assays from IDT were used: Irf-3: Rn.PT.47.14307929; Irf-7: Rn.PT.47.7921156.
Cell survival. Neuronal monolayers were mock-infected or infected with SINV (MOI=5) or VEEV (MOI=50). Cell viability was determined microscopically by trypan blue exclusion at the indicated times after infection and reported as percent viable.

Protein radiolabeling. At the indicated times after infection, the neuronal monolayer growth medium was replaced with 1X PBS (pH 6.2) containing 50 µCi of Trans [35S]-Label (1175 Ci/mmol, methionine 70%; cysteine ≤15%, MP Biomedical, LLC) per ml. After 1 h, the cells were dislodged, collected by centrifugation, and lysed with cold RIPA buffer. The lysates were subjected to SDS-PAGE and autoradiography.

Transfection of dsRNA. Four days into the differentiation process, RNA duplexes (40 pmol total) specific to three nonoverlapping regions of the cDNA of interest (IDT trifecta RNA) were transfected into AP-7 neurons using RNAiMAX (Invitrogen). The neurons were infected 96 h after transfection.

Accession Numbers. Irf-3:NM_001006969; Irf-7:NM_001033691.

RESULTS

Alphavirus multiplication is restricted in differentiated neurons. To define AP-7 neurons as a faithful model of in vivo infection, we first compared alphavirus multiplication in immature cAP-7 and mature dAP-7 neurons. After differentiation of AP-7 cells for 7 days, cell division ceases, processes extend from the cell body, and neuronal markers are expressed by the mature dAP-7 neurons (50). cAP-7 neurons produced new infectious SINV by 6 h after infection with an increase to $10^{8.6}$ pfu/mL by 18 h (Fig. 1A). In contrast, dAP-7 neurons did not produce new SINV until 9 h and virus titers increased more slowly to a 50-fold lower peak of $10^{6.9}$ pfu/mL at 18 h (Fig 1A).

Production of infectious virus then continued at the same levels through 48 h for both cAP-7 and dAP-7 neurons.

Infectious virus production by the New World alphavirus VEEV (TC-83 strain)
was similarly affected by neuronal differentiation. In cAP-7 neurons, VEEV accumulated through 24 h with peak titers of $10^{7.8}$ pfu/mL (Fig. 1B). In contrast, VEEV production by dAP-7 neurons was not detectable until 12 h and increased only modestly through 24 h with a peak titer of $10^{5.2}$ pfu/mL.

Additionally, mature neurons were less susceptible to infection with VEEV than SINV. By 8 h after with SINV at an MOI of 5, more cAP-7 cells (58%) were positive for E2 glycoprotein than dAP-7 cells (28%) as determined by immunofluorescence microscopy (Fig. 1C). However, similar numbers of cAP-7 and dAP-7 neurons were positive by 24 h after infection. Upon infection at an MOI of 50, fewer dAP-7 (52%) than cAP-7 (84%) neurons were positive by 8 h after infection, but levels of infection (>90%) were similar 24-48 h after infection. This indicates that all cells in culture were susceptible to SINV infection regardless of maturation state. Immature neurons were susceptible to VEEV at either MOI tested, with greater than 90% of cells infected by 24 h after infection. In contrast, only 40% of dAP-7 neurons were positive for E2 glycoprotein through 48 h after VEEV infection at an MOI of 5. At an MOI of 50, 70% of dAP-7 neurons were infected by 8 h after infection and greater than 90% by 24 h. This suggests that maturation of dAP-7 neurons confers protection to VEEV infection. Therefore, studies using VEEV were conducted at an MOI 50 to ensure infection of all cells and to focus on factors restricting intracellular replication. During both SINV and VEEV infection, cAP-7 neurons displayed a brighter fluorescent signal at 8 h after infection than dAP-7 neurons, suggesting greater synthesis of glycoprotein on a per cell basis.

A defect in any step of virus replication or in assembly of virus particles could reduce infectious virus production. To determine if progeny virus from dAP-7 neurons was morphologically distinct from that produced by cAP-7 neurons, we imaged budding virus 24 h after infection using transmission electron microscopy (TEM). The majority of virions produced by both cAP-7 and dAP-7 neurons were single particles with only
occasional observation of multiple particles packaged into one envelope (Fig. 2A). Virus particles from both cAP-7 and dAP-7 neurons were comparable in size and electron density indicating that virion assembly was not likely to be responsible for reduced infectious virus production.

To compare viral RNA synthesis between cycling and differentiated AP-7 neurons, viral RNA was measured using qRT-PCR (Fig. 2B). The synthesis and accumulation of viral RNA was slower in dAP-7 neurons. However, levels of viral RNA at 24 h were similar in cAP-7 and dAP-7 neurons potentially due to decreased infectious virus production. Delayed SINV RNA synthesis in dAP-7 neurons may reduce the amount of genomic RNA available to be packaged into new virions or the RNA available for translation of viral proteins needed for virion assembly.

SINV nonstructural proteins (nsP1-4) are translated from both incoming viral genomic RNA and newly synthesized full-length, positive-strand RNA. Structural proteins (C, E1, pE2, 6K) are translated from a subgenomic mRNA produced later in infection. Thus, delayed RNA replication could also affect the timing and levels of viral protein production. Comparison of SINV protein production in cAP-7 and dAP-7 neurons showed a delay in production of both nonstructural and structural proteins by dAP-7 cells (Fig. 2C). nsP3 was detected in cAP-7 neurons at 4 h after infection and increased through 8 h but was not detectable until 12 h in dAP-7 neurons (Fig. 2C top).

The envelope glycoprotein E2 is produced as a precursor pE2 (54 kDa) that is cleaved to E2 (47 kDa) and E3 (7 kDa) prior to virion maturation. In cAP-7 neurons, pE2 was detected by 5 h after infection with cleavage to E2 detected at 7 h with both pE2 and E2 accumulating in similar amounts through 24 h (Fig. 2C middle). In dAP-7 neurons, neither pE2 nor E2 were detected until 24 h after infection. Therefore, multiple steps of SINV multiplication are delayed in differentiated neurons, all of which probably contribute to a reduced production of infectious virus.
Cell survival after alphavirus infection is extended in differentiated neurons. In addition to reduced virus multiplication in vivo, host maturation is associated with decreased morbidity and mortality upon SINV TE and VEEV TC-83 infection in mice (25, 26, 53). SINV induces apoptosis of immature neurons both in vivo and in cultured cells (58-61). Cytopathic effects (CPE), including cell rounding, detachment and ultimately production of cytoplasmic blebs, were first apparent between 12 and 18 h in both SINV- and VEEV-infected cAP-7 neurons. Two days after infection less than 10% of cAP-7 neurons were viable, as determined by trypan blue exclusion (Fig. 3). In contrast, 62% of SINV-infected and 73% of VEEV-infected dAP-7 neurons were viable at this time and reduction in survival to 10% did not occur until 4 d after infection. Therefore, AP-7 differentiation extended survival after alphavirus infection, potentially the result of reduced virus replication, increased cellular survival signaling, or a combination of both.

Shut-off of host protein synthesis is delayed upon infection of differentiated neurons. Alphavirus infection causes significant changes in cellular processes including inhibition of host protein synthesis. To determine if effects on host translation differ during SINV or VEEV infection of cAP-7 and dAP-7 neurons, we monitored incorporation of radiolabelled Cys/Met throughout infection (Fig. 4). In SINV-infected cAP-7 neurons, reduced host protein synthesis was evident by 6 h and most translation of cellular proteins was shut off by 12 h. In contrast, in SINV-infected dAP-7 neurons shut off of host protein synthesis occurred between 12 and 24 h and correlated with delayed nsP3 synthesis (Fig. 2D). Compared to SINV infection, translational arrest induced by VEEV infection of cAP-7 neurons was slower, occurring between 24 and 48 h after infection, although induction of cell death was similar (Fig. 3). During VEEV infection of dAP-7 neurons, host protein synthesis was minimally affected through 48 h. In agreement with
immunoblot studies (Fig. 2D), structural proteins were visible by 6 h in SINV- and VEEV-infected cAP-7 neurons, but were not apparent until 24 h after infection in dAP-7 neurons. Therefore, changes in AP-7 neurons associated with differentiation delayed alphavirus-mediated translational arrest potentially providing a longer window for synthesis of host antiviral proteins after infection.

Bunyavirus replication is reduced in differentiated AP-7 neurons. To determine if dAP-7 cell-mediated restriction of virus multiplication was unique to alphaviruses or a more general phenomenon for neuronotropic viruses, we assessed progeny virus production of the unrelated encephalitic La Crosse virus (LACV, original strain; family bunyaviridae). At an MOI of 5, LACV was produced rapidly in cAP-7 neurons with peak titers of $10^{6.5}$ pfu/mL at 6 h after infection (Fig. 5). In dAP-7 neurons, LACV titers increased less than 10 fold in 24 h. We were unable to test the effect of higher MOIs for LACV infection due to low stock titers and cannot rule out that LACV would replicate somewhat better with infection of dAP-7 neurons at a higher MOI. Overall, these studies indicate that neuronal differentiation confers inherent characteristics that restrict replication of three different neuronotropic viruses.

AP-7 differentiation is associated with the upregulation of antiviral gene expression. We hypothesized that neurons may upregulate expression of cellular defense genes during differentiation, allowing an expedited innate immune response upon pathogen detection. We compared the gene expression of 84 antiviral genes in uninfected dAP-7 neurons to that in uninfected cAP-7 neurons using an antiviral response qPCR array. Expression of 22 genes were upregulated 2 fold or more during differentiation (Table 1). These included genes at all stages of the innate immune signaling pathway including the dsRNA receptors tlr3 (11.78) and ddx58/rig-I (3.65),

Bunyavirus replication is reduced in differentiated AP-7 neurons. To determine if dAP-7 cell-mediated restriction of virus multiplication was unique to alphaviruses or a more general phenomenon for neuronotropic viruses, we assessed progeny virus production of the unrelated encephalitic La Crosse virus (LACV, original strain; family bunyaviridae). At an MOI of 5, LACV was produced rapidly in cAP-7 neurons with peak titers of $10^{6.5}$ pfu/mL at 6 h after infection (Fig. 5). In dAP-7 neurons, LACV titers increased less than 10 fold in 24 h. We were unable to test the effect of higher MOIs for LACV infection due to low stock titers and cannot rule out that LACV would replicate somewhat better with infection of dAP-7 neurons at a higher MOI. Overall, these studies indicate that neuronal differentiation confers inherent characteristics that restrict replication of three different neuronotropic viruses.

AP-7 differentiation is associated with the upregulation of antiviral gene expression. We hypothesized that neurons may upregulate expression of cellular defense genes during differentiation, allowing an expedited innate immune response upon pathogen detection. We compared the gene expression of 84 antiviral genes in uninfected dAP-7 neurons to that in uninfected cAP-7 neurons using an antiviral response qPCR array. Expression of 22 genes were upregulated 2 fold or more during differentiation (Table 1). These included genes at all stages of the innate immune signaling pathway including the dsRNA receptors tlr3 (11.78) and ddx58/rig-I (3.65),
transcription factors stat1 (5.35) and Irf-7 (3.59), and effector genes ifnb1 (4.67) and isg15 (5.91). Expression of two genes, cxcl10 and spp1, were reduced more than 2-fold during differentiation.

As STAT-1 is an important determinant for outcome after SINV infection (21), we determined if protein levels were increased in AP-7 neurons during differentiation. STAT-1 levels were higher in uninfected dAP-7 than cAP-7 neurons and remained unchanged in both during infection (Fig. 6A). Classically, activation of downstream signaling through the JAK/STAT pathway requires phosphorylation of STAT1. Thus, to compare signaling in undifferentiated and differentiated AP-7 neurons we investigated the phosphorylation state of STAT1. Phosphorylated STAT1 (Y701) was not detected before or after SINV infection (Fig. 6A), although the cells responded to recombinant IFN treatment with STAT1 phosphorylation (Fig. 6B). This confirmed previous studies showing a lack of STAT1 phosphorylation during SINV infection of AP-7 or CSM neurons (62).

Differentiated neurons upregulate IRF-7 expression. IRFs compose a family of transcription factors that upon activation regulate expression of IFN and ISG mRNAs. IRF-3 and IRF-7 are central regulators of the cellular innate immune response and the convergence point for integration of pathogen detection pathways. IRF-3 is constitutively expressed and found inactive in the cytoplasm whereas IRF-7 oftentimes is produced in response to IFN signaling. Upon activation, IRF-3 and IRF-7 are phosphorylated, translocate to the nucleus, and stimulate ISG expression. In a positive feedback loop, IFN upregulates expression Irf-3 and Irf-7 mRNAs. We compared expression of Irf-3 and Irf-7 during AP-7 differentiation using qRT-PCR (Fig. 7A). Day 0 corresponds to cAP-7 neurons as these samples were not supplemented with differentiation media nor were they incubated at the nonpermissive temperature. Gene
expression throughout the time course is represented as the fold-change compared to
day 0 levels. *Irf-3* mRNA levels increased steadily through the differentiation process
and were 4-fold higher on day 7. *Irf-7* mRNA levels increased 13 fold on day 3 and were
sustained at an elevated level with an average 11-fold increase on day 7.

We confirmed the effect of differentiation on *Irf* expression using CSM14.1
neurons, an immortalized rat nigral neuron cell-line that restricts SINV infection similarly
to dAP-7 neurons when differentiated (15) (Fig. 7B). Similar fold increases in *Irf-3* and
*Irf-7* transcripts were detected in dCSM14.1 neurons compared to cCSM14.1 cells.

These studies show that expression of *Irf-3* and *Irf-7* mRNAs are upregulated during
differentiation of two distinct neuronal cell cultures.

We investigated the levels of IRF-3 and IRF-7 protein in cycling and differentiated
AP-7 neurons (Fig. 7C). No changes in IRF-3 protein levels were detected during
differentiation. At day 0, a single IRF-7 band was visible by immunoblot analysis. Upon
differentiation, a slower mobility band accumulated and a third band of intermediate
mobility was visible upon longer exposures. These bands correspond in size to mouse
IRF-7 isoforms α, β, and γ (63). All three isoforms contain exons 8 and 9 and so the
corresponding mRNAs would be detected by qRT-PCR (Fig. 7A, B). In dCSM14.1
neurons we detected a single IRF-7 band corresponding in size to the α isoform, while
no IRF-7 was detected in cCSM14.1 neurons (Fig. 7D). Importantly, IRF-7 α, the full-
length isoform expressed by differentiated neurons, is a potent transactivator whereas
IRF-7 γ, the primary isoform expressed by undifferentiated neurons inhibits IRF-7 α/β
transactivation (63). Thus, in two distinct neuronal cell lines total IRF-7 protein levels are
increased upon differentiation, in agreement with increased mRNA levels, and
differentiation is associated with increased levels of the active full-length α isoform of
IRF-7.
To investigate whether IRF-3 or IRF-7 levels were changed during development in vivo, we compared IRF-3 and IRF-7 in brain homogenates from 3-day and 6-week-old C57BL/6J mice. In 3-day-old mice, IRF-3 protein migrated as would be expected for a predicted molecular weight of 62 kDa. However, IRF-3 was detected in a high molecular weight complex in the brains of 6 week old mice, suggesting that it is in a tight complex that was not dissociated by SDS-βME and high heat. In brain homogenates from 3-day-old mice, IRF-7 was predominately in the γ isoform. In contrast, the α isoform was dominant in brain homogenates from 6-week-old mice. Thus, the IRF-7 isoforms switched from the dominant negative γ isoform to the active α isoform upon maturation of cultured neurons and in the mouse brain, possibly priming neurons for a rapid response upon infection.

Because dAP-7 neurons had decreased SINV multiplication (Fig. 1A) and increased levels of Irf-3 and Irf-7 expression (Fig. 7B), we assessed whether media from uninfected dAP-7 neurons could confer these characteristics on cAP-7 neurons. cAP-7 neurons were incubated for 24 h before infection with normal media (DMEM+10% FBS), differentiation media (normal media plus dopamine, insulin and ascorbic acid) or conditioned differentiation media from 7-9 day dAP-7 neurons (Fig. 8A). No differences (one-way ANOVA) in SINV multiplication were detected. Additionally, pretreatment did not alter expression of either Irf-3 or Irf-7. Thus, the antiviral properties observed in dAP-7 neurons could not be conferred to cAP-7 neurons by the differentiation media alone or by soluble factors produced by dAP-7 neurons.

IRF-3 and IRF-7 expression is increased after infection of dAP-7 neurons.

In many cell types, activation of innate immune signaling results in increased Irf-7 gene expression. Thus, we compared Irf-3 and Irf-7 gene expression levels in cAP-7 and dAP-7 cells following infection with SINV, VEEV and LACV. Irf-3 and Irf-7 gene
expression increased less than 3-fold after infection of cAP-7 neurons with SINV, VEEV or LACV (Fig. 9A, B). In contrast, \textit{Irf-3} and \textit{Irf-7} gene expression was rapidly and substantially increased in dAP-7 neurons after infection (Fig. 9A, B). By 1 h after infection with all three viruses, \textit{Irf-3} was increased 10-fold and \textit{Irf-7} was increased approximately 25-fold. \textit{Irf-3} continued to increase through 24 h after SINV infection while VEEV and LACV-infected dAP7 cells did not show further increases (Fig. 9A). \textit{Irf-7} expression was further increased at 24 h after infection with all three viruses (Fig. 9B). To determine if increased mRNA levels resulted in increased protein levels, we investigated protein accumulation in SINV-infected cells by immunoblot analysis. In cAP-7 neurons, total levels of IRF-7 protein increased during infection (Fig 9C left). However, the isoform that increased was the dominant negative $\gamma$ form, indicating that even after infection, cAP-7 neurons do not produce the functional $\alpha$ or $\beta$ IRF-7 isoforms. No change in total IRF-7 protein levels was detected in dAP-7 neurons during SINV infection and isoform $\alpha$ remained the most abundant form throughout the time course (Fig. 9C right). IRF-7 is reported to have a short-half life of approximately 30 min in most cells, but the half-life in neurons is unknown and virus infection has a variable effect on IRF-7 stability (64). Therefore, increased gene expression may be necessary to maintain IRF-7 protein levels during infection.

To determine if IRF-3 and IRF-7 were critical for restricting virus multiplication during alphavirus infection of dAP-7 cells, we silenced \textit{Irf-3} and \textit{Irf-7} alone or together during SINV and VEEV infection of dAP-7 neurons. dsRNA was transfected into AP-7 neurons on day 4 of differentiation. Replacement of differentiation media at this stage did not affect the neurons morphologically or the restriction of virus multiplication (Fig. 10D, E, none versus dAP-7). In uninfected neurons, \textit{Irf-3} expression was decreased 8 to 71-fold when silenced alone and 7 to 53-fold when silenced along with \textit{Irf-7} (Fig. 10A). \textit{Irf-7}
expression was decreased 25 to 42-fold when silenced alone and 15 to 36-fold when silenced with Irf-3 (Fig 10B).

The level of silencing was then determined during infection. Compared to neurons that had not been transfected with silencing RNA, Irf-3 expression alone was decreased 28-fold during SINV infection and 72-fold during VEEV infection. When silenced along with Irf-7, Irf-3 levels were further decreased 26 to 111-fold during alphavirus infection (Fig. 10A). IRF-3 protein levels were decreased to the level of detection when silenced alone or with Irf-7 (Fig. 10C middle). When silenced alone, Irf-7 mRNA levels decreased 11-fold after SINV infection and 19-fold when co-silenced with Irf-3 (Fig. 10B). After VEEV infection there was a 29 to 33-fold decrease in Irf-7 mRNA levels compared to untransfected dAP-7 neurons when silenced alone or with Irf-3.

However, IRF-7 protein was still detectable regardless of virus infection or silencing conditions (Fig. 10C top) and we were unable to identify an RNA silencing strategy that consistently reduced IRF-7 protein levels at the time of or through out infection. It is possible that IRF-7 in a stable transcriptionally active complex is less susceptible to rapid turnover. Levels of Irf-3 and Irf-7 mRNAs changed less than 4-fold in neurons transfected with irrelevant dsRNAs, indicating that neither transfection nor media changes affected expression.

To determine the impact of Irf-3 and Irf-7 dsRNA on virus multiplication in dAP-7 neurons, we compared the levels of infectious SINV and VEEV produced by dAP-7 neurons with and without Irf silencing prior to infection (Fig. 10 D,E). There was no effect on replication of either virus when Irf-7 transcript levels were decreased alone or simultaneously with Irf-3. Furthermore, reduction in IRF-3 protein alone did not increase production of either SINV or VEEV compared to untreated dAP-7 neurons (one-way ANOVA). Thus, loss of IRF-3 is not sufficient to reverse restriction of alphavirus replication in differentiated AP-7 neurons.
DISCUSSION

Host maturity is an important factor determining the outcome of viral encephalitis (1, 3-9). We have previously demonstrated maturation-dependent restriction of SINV replication in CSM14.1 neuronal cells (15). In this study we have shown maturation-dependent restricted replication of the encephalitic arboviruses SINV, VEEV, and LACV in olfactory bulb-derived AP-7 neurons. During SINV infection, maturation did not affect neuronal susceptibility to infection or virion maturation. However, maturation did reduce susceptibility to VEEV infection. When equal percentages of immature and mature neurons were infected with either alphavirus, restriction was associated with delayed synthesis of viral RNA and protein, decreased production of infectious virus, delayed shutoff of host protein synthesis, and improved viability of infected neurons. During maturation from immature cycling cells to differentiated neurons, expression of Stat-1 and the immunoregulatory transcription factors Irf-3 and Irf-7 was increased. The IRF-7 protein isoforms changed from production of the inhibitory γ short form to the transcriptionally competent α long form. Silencing of Irf-3 and Irf-7 did not improve viral replication in mature neurons indicating that there are additional maturation-dependent mechanisms for restriction of virus replication. However, we were unable to consistently reduce IRF-7 protein levels by RNA-silencing, so IRF-7 may yet prove to be important for restricting virus replication.

To counter host antiviral signaling, viruses encode IFN antagonists that circumvent the host antiviral response by distinct mechanisms, often by inhibiting host transcription and translation (65). SINV nsP2 shuts off both host transcription and translation whereas VEEV capsid is responsible for transcriptional arrest (66-68). LACV NSs inhibits RNA polymerase II-dependent transcription to block IFN signaling (69).
our studies, host protein synthesis was sustained longer during infection of dAP-7
neurons as compared to that in cAP-7 neurons during alphavirus infection. Additionally,
alphavirus protein synthesis was delayed in dAP-7 neurons. Thus, active translation of
antiviral proteins in dAP-7 neurons could likely support survival and reduce virus
multiplication prior to accumulation of viral proteins required for cellular translation arrest.

The protective role of IFNs against encephalitic virus infections has been
demonstrated in other in vitro systems and in vivo. Pretreatments of a variety of cultured
cells, including neurons, with IFN-α, -β, or -γ prevents more than 99% of infectious SINV
production, significantly reduces synthesis of viral proteins, and preserves cellular
viability and protein synthesis (20, 62, 70). The G3A mutation in VEEV TC-83 renders
this strain responsive to IFN-α/β priming and correlates with decreased mortality in
weanling mice (25, 26, 70). Mice that lack the α-chain of the IFN-α/β receptor, IFN-β, or
the IFN-activated STAT1 transcription factor are more susceptible to SINV, VEEV TC-
83, and LACV (21, 24, 26, 27, 70, 71). Subprotective levels of IFN-β are sufficient to
stimulate antiviral signaling during SINV infection and allow for signaling during the first
hours of infection which determine the outcome of infection (23). Consistent with these
previous studies, STAT1 was not phosphorylated in either dAP-7 or cAP-7 neurons and
transfer of dAP-7-dervied media to cAP-7 neurons failed to confer protection, suggesting
that if IFN were present, it was not signaling detectably through the classical JAK/STAT
pathway.

Canonically, IRFs activate ISG expression in response to IFN as well as
stimulate IFN expression. However, the control of innate immune signaling varies
according to tissue and virus (72-75). Increased susceptibility of IRF-7-deficient mice to
viruses such as West Nile virus, Chikungunya virus, and SINV demonstrates the central
importance of IRF-7 in orchestrating IFN signaling (44, 47, 76-78). Noncanonically, IRF-
1, -3, and -7 can upregulate ISG expression in the absence of measurable IFN (48, 79-82). Utilization of noncanonical, STAT1-independent antiviral signaling by neurons has been demonstrated in neurons during measles virus, WEEV, and St. Louis encephalitis virus infection (49, 83). These studies support the importance of higher levels of *Irf-7* and *Irf-3* to enable differentiated neurons to orchestrate a prompt and effective antiviral response. Additionally, *Irf-7* is constitutively expressed in the brains of naïve, weanling mice and is upregulated in neurons during LACV infection (28). By analyzing protein as well as mRNA, we found that distinct IRF-7 isoforms are produced in brains of newborn and weanling mice, with the functional α isoform prominent in weanling mice.

Our studies are consistent with a model in which pre-assembled IRFs mediate rapid intracellular signaling upon pathogen detection to induce the antiviral response. Production of a distinct isoform of IRF-7 may contribute to the improved IFN response in differentiated rodent neurons. Conversely, the presence of the dominant negative isoform in immature rodent neurons may contribute to the failure of these cells to mount an antiviral response. In the absence of *Irf-3* and *Irf-7*, and presumably consequent ISG expression, viral titers did not increase to those measured in cAP-7 neurons. We cannot rule out that residual IRF-7 was sufficient to support antiviral signaling during infection. However, IRF-mediated signaling may play a greater role in vivo to control virus spread, orchestrate virus clearance, or modulate the adaptive immune response in the CNS.

Recently, advances have been made in understanding the active role neurons play in the response to neuronotropic virus infection. Along with our studies, these findings collectively suggest that the CNS has evolved specialized control of the cellular response to pathogens, possibly due to the limited regenerative properties and specific function of neurons. Collectively, maturation of neurons is associated with increased levels of IFN signaling pathway components (33, 34). Mature neurons are able to produce an innate immune response upon virus infection (28, 30-33). Increased basal
levels of Irf-3 and Irf-7 expression could contribute to an enhanced host response to infection. However, innate immune signaling does not fully explain the improved outcome for mature neurons following virus infection (33, 45). Further comparison of antiviral gene expression in mature and immature neurons may identify other factors that make greater contributions to age-dependent susceptibility to encephalitic viruses.

ACKNOWLEDGMENTS

These studies were funded in part by research grant R01 NS038932 (DEG) and training grants T32 AI007247 (KLWS) and T32 GM07309 (PSV) from the National Institutes of Health.

We thank Drs. Andrew Pekosz, Wei Li, and Kirsten Kulscar for many helpful discussions.

We acknowledge help from the Johns Hopkins microscopy core and production of anti-E2 monoclonal antibody 209 by the Johns Hopkins University School of Medicine Department of Neuroscience Monoclonal Antibody Core (NS050274).

REFERENCES


7. Sigel MM. 1952. Influence of age on susceptibility to virus infections with
607.
neurotropic virus and its changing distribution in the developing brain is a function
resistance to lethal alphavirus encephalitis in mice: analysis of gene expression in
the central nervous system and identification of a novel interferon-inducible
16. Binder GK, Griffin DE. 2003. Immune-mediated clearance of virus from the
1631.
induction, signalling, antiviral responses and virus countermeasures. Journal of
General Virology 89:1–47.
Sindbis virus-infected cells is restored by anti-E2 monoclonal antibody treatment.
22. Müller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet
Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is
TM, Trent DW. 1993. Attenuation of Venezuelan equine encephalitis virus strain
TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. J
interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an


Bulb S, Park S, Budhirajd M, Desnoyers S, Montrose MH, Poirier GG.


Table 1: Gene products with ≥2 fold change in expression in dAP-7 neurons compared to cAP-7 neurons

<table>
<thead>
<tr>
<th>gene</th>
<th>reference</th>
<th>fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd80</td>
<td>NM_012926</td>
<td>152.58</td>
</tr>
<tr>
<td>Il6</td>
<td>NM_012589</td>
<td>95.22</td>
</tr>
<tr>
<td>Casp1</td>
<td>NM_012762</td>
<td>33.48</td>
</tr>
<tr>
<td>Nlrp3</td>
<td>XM_220513</td>
<td>14.63</td>
</tr>
<tr>
<td>ctsb (Cathepsin B)</td>
<td>NM_022597</td>
<td>14.36</td>
</tr>
<tr>
<td>Tlr3</td>
<td>NM_198791</td>
<td>11.78</td>
</tr>
<tr>
<td>Dhx58 (LGP2)</td>
<td>NM_001106645</td>
<td>10.11</td>
</tr>
<tr>
<td>Azi2</td>
<td>NM_001025705</td>
<td>9.45</td>
</tr>
<tr>
<td>Tlr9</td>
<td>NM_198131</td>
<td>8.62</td>
</tr>
<tr>
<td>Isg15</td>
<td>XM_216605</td>
<td>5.91</td>
</tr>
<tr>
<td>Stat1</td>
<td>NM_032812</td>
<td>5.35</td>
</tr>
<tr>
<td>Il18</td>
<td>NM_019165</td>
<td>4.90</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Log2 Fold Change</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Atg12</td>
<td>NM_001038495</td>
<td>4.69</td>
</tr>
<tr>
<td>Ifnb1</td>
<td>NM_019127</td>
<td>4.67</td>
</tr>
<tr>
<td>Ira4</td>
<td>NM_001106791</td>
<td>4.26</td>
</tr>
<tr>
<td>cts5 (Cathepsin S)</td>
<td>NM_017320</td>
<td>3.90</td>
</tr>
<tr>
<td>cts11 (Cathepsin L1)</td>
<td>NM_013156</td>
<td>3.77</td>
</tr>
<tr>
<td>Ddx58 (RIG-I)</td>
<td>NM_001106645</td>
<td>3.65</td>
</tr>
<tr>
<td>Irf7</td>
<td>NM_001033691</td>
<td>3.59</td>
</tr>
<tr>
<td>Iil15</td>
<td>NM_013129</td>
<td>3.52</td>
</tr>
<tr>
<td>Tbkbp1</td>
<td>NM_172021</td>
<td>3.37</td>
</tr>
<tr>
<td>Dak</td>
<td>NM_001039031</td>
<td>3.10</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>NM_139089</td>
<td>-4.64</td>
</tr>
<tr>
<td>Spp1</td>
<td>NM_012881</td>
<td>-4.35</td>
</tr>
</tbody>
</table>

**FIGURE LEGENDS**

**FIG. 1.** Alphavirus multiplication is restricted in differentiated AP-7 neurons. cAP-7 (dashed lines) or dAP-7 (solid lines) neurons were infected with the (A) TE strain of SINV (MOI 5) or (B) TC-83 strain of VEEV (MOI 50). Viral titers in supernatant fluids collected at the indicated times after infection were measured by plaque formation in BHK cells and are expressed as the mean log_{10} PFU/ml ± standard deviation of triplicate samples from a representative of three experiments. (C) Percentages of cAP-7 and dAP-7 neurons expressing detectable E2 glycoprotein at the indicated times after infection were determined by immunofluorescence. E2-positive cells compared to the total number of DAPI-positive cells were determined in three, nonoverlapping fields.

**FIG. 2.** SINV intracellular replication is delayed in differentiated AP-7 neurons. AP-7 neurons were infected with the TE strain of SINV (MOI 5). (A) cAP-7 and dAP-7 neurons were fixed at 24 h after infection and ultrastructure analysis was performed by transmission electron microscopy of thin sections. Bar = 100 nM. (B) Total cellular RNA was collected at various times after infection; cDNA was produced using SINV-specific primers and quantified by qPCR compared to standard SINV genomic DNA.
levels in cAP-7 (dashed lines) or dAP-7 (solid lines) are expressed as the mean SINV copy number (log_{10}) ± standard deviation of triplicate samples from a representative of two experiments. (C) Immunoblot analysis of total cell lysates prepared at the indicated times from mock-infected (M) or SINV-infected cAP-7 (left) or dAP-7 (right) neurons with anti-nsP3 (top), anti-E2 (middle) or anti-β-actin (bottom) antibodies. A representative of 2 experiments is shown. Significant differences between cAP-7 and dAP-7 at each time point determined by two-way ANOVA with Bonferroni’s post-test are shown (***, P < 0.001).

FIG. 3. Virus-induced cell death is delayed in differentiated AP-7 neurons. cAP-7 (dashed lines) and dAP-7 (solid lines) neurons were mock-infected or infected with either SINV (MOI 5) or VEEV (MOI 50). Live cells, determined by trypan blue exclusion, were counted at the indicated days after infection. Surviving cells are expressed as a percentage of the mock-infected cells at the corresponding time point ± standard deviation of triplicate samples. Representative data from three independent experiments are presented.

FIG. 4. Alphavirus-mediated shut-off of host protein synthesis is delayed in differentiated AP-7 neurons. Mock-, SINV (MOI 5) - or VEEV (MOI 50) -infected cAP-7 (upper) and dAP-7 (lower) neurons were radiolabelled for 1 h with [^{35}S]-labeled cysteine/methionine at the indicated times after infection. Protein synthesis (equal cpm per lane) was analyzed by SDS-PAGE autoradiography. Viral proteins are denoted to the right. A representative of three experiments is shown.

FIG. 5. Differentiated AP-7 neurons also restrict multiplication of LACV. cAP-7 (dashed lines) or dAP-7 (solid lines) neurons were infected with the original strain of
LACV (MOI 5). Viral titers in supernatant fluids collected at the indicated times after infection were measured by plaque formation on BHK cells and are expressed as the mean log$_{10}$ PFU/ml ± standard deviation of triplicate samples from a representative of two experiments. Significant differences between cAP-7 and dAP-7 at each time point determined by two-way ANOVA with Bonferroni’s post-test are shown (***, $P < 0.001$).

**FIG. 6.** Stat1 is not phosphorylated during SINV infection of AP-7 neurons. (A) cAP-7 or dAP-7 neurons were mock-infected (M) or infected with SINV (MOI 5). Total cell lysates prepared at the indicated times after infection were analyzed with anti-pSTAT1 (P-Y701) (top row), anti-STAT1 (middle row) or α-β-actin (bottom row) antibodies. A representative of 2 experiments is shown. (B) cAP-7 or dAP-7 neurons were treated with 500 IU/mL of rIFN α, β, or γ. Total cell lysates prepared 4 h after treatment were analyzed by immunoblot using anti-STAT1 (P-Y701) (top row) or anti-β-actin (bottom row) antibodies. A representative of 2 experiments is shown.

**FIG. 7.** Irf-3 and Irf-7 mRNAs and IRF-7 protein increase during differentiation. (A) Total cellular RNA was collected at various times after plating of AP-7 neurons. Neurons were incubated at the permissive temperature in DMEM + 10% FBS, one day prior to day 0 collection; corresponding to cAP-7 neurons. After day 0, neurons were maintained in differentiation media at the nonpermissive temperature as described for the differentiation procedures. cDNA was produced using random primers, Irf-3 and Irf-7 mRNAs were measured by qPCR and normalized to GapDH levels. mRNA levels are expressed as the mean value compared to levels in uninfected day 0 cAP-7 neurons ± standard deviation of six samples. (B) Total RNA was collected from day 0 (9 replicates) and day 7 (12 replicates) AP-7 neuron cultures or from triplicate cultures of day 0 or day 7 AP-7 neurons and the cDNA was produced using random primers. Irf-3 and Irf-7 mRNAs were measured by qPCR and normalized to GapDH levels. mRNA levels are expressed as the mean value compared to levels in uninfected day 0 cAP-7 neurons ± standard deviation of six samples.
28 CSM14.1 neurons. mRNA levels, measured as in A, are expressed as the mean value of differentiated cultures compared to levels in day 0 AP-7 or CSM14.1 neurons ± standard deviation. (C) Total cell lysates prepared from AP-7 neurons during differentiation were analyzed by immunoblot analysis with anti-IRF-7 (top row), anti-IRF-3 (middle row) or anti-β-actin (bottom row) antibodies. Multiple isoforms of IRF-7 are detected by the IRF-7 antibody. (D) Total cell lysates prepared from CSM neurons at the indicated days during differentiation were analyzed by immunoblot analysis with anti-IRF-7 (top row) or anti-β-actin (bottom row) antibodies. (E) 10% brain homogenates from 3-day or 6-week-old C57BL/6J mice were analyzed by immunoblot analysis with anti-IRF-7 (top row), anti-IRF-3 (middle row) or anti-β-actin (bottom row) antibodies. A representative of at least 2 experiments is shown. Significant differences between cAP-7 and dAP-7 at each time point determined by one-way ANOVA with Dunnett’s post-test are shown (***, P < 0.001).

FIG. 8. Media exchange does not confer antiviral properties. cAP-7 neurons were incubated in normal media (DMEM + 10% FBS), differentiation media or supernatant fluids from dAP-7 neurons for 24 h. (A) Extracellular virus collected at 5 and 24 h after SINV infection (MOI 5) infection was measured by plaque assay. Titers are expressed as the mean log$_{10}$ PFU/ml ± standard deviation of triplicate samples. (B) Total cellular RNA was collected and cDNA was produced using random primers. Gene expression was measured by qPCR and normalized to GapDH levels. RNA levels are expressed as the mean value compared to levels in cAP-7 neurons in normal media ± standard deviation of six samples. Significant differences by Student’s t-test or one-way ANOVA with Dunnett’s post-test are shown (**, P < 0.01).
FIG. 9. Expression of Irf-3 and Irf-7 is upregulated during infection of differentiated AP-7 neurons. cAP-7 or dAP-7 neurons were infected with SINV (MOI 5), VEEV (MOI 50), or LACV (MOI 5). Total cellular RNA was collected at the 1, 12 or 24 h after infection and cDNA was produced using random primers. Irf-3 (A) or Irf-7 (B) gene expression was measured by qPCR and normalized to GapDH levels. RNA levels are expressed as the mean value compared to levels in mock-infected cAP-7 neurons ± standard deviation of triplicate samples. A representative of 2 experiments is shown. Significant differences by two-way ANOVA with Bonferroni’s post-test are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001). (C) cAP-7 or dAP-7 neurons were mock infected or infected with SINV (MOI 5). Total cell lysates were prepared at the indicated time after infection and analyzed by immunoblot using anti -IRF-7 (top) or anti -β-actin (bottom) antibodies. A representative of 2 experiments is shown.

FIG. 10. Virus replication is unchanged upon silencing of IRF-3 or IRF-7 during infection of differentiated AP-7 neurons. On day 4 of differentiation dAP-7 neurons were transfected with dsRNA specific to irf-3 (3), irf-7 (7), both (3/7), or egfp (E), or not transfected, with media change (N). For comparison, dAP-7 neurons were differentiated as normal, without media changes (d). 96 h after transfection, dAP-7 neurons were infected with SINV (MOI 5) or VEEV (MOI 50). Total cellular RNA was collected at 0 or 24 h after infection and cDNA was produced using random primers. Irf-3 (A), Irf-7 (B), gene expression was measured by qPCR and normalized to GapDH levels. RNA levels are expressed as the mean value compared to levels in mock-infected, 0h dAP-7 neurons ± standard deviation of duplicate samples. Significant differences by one-way ANOVA with Dunnet’s post-test are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001). (C) Total cell lysates were prepared at the indicated time after infection and analyzed by
immunoblot using anti-IRF-7 (top), anti-IRF-3 (middle), or anti-β-actin (bottom) antibodies. Viral titers in supernatant fluids collected at the indicated times after SINV (D) or VEEV (E) infection were measured by plaque formation in BHK cells and are expressed as the mean Log_{10} PFU/ml ± standard deviation of triplicate samples. No significant differences by one-way ANOVA with Dunnet's post-test. A representative of 2 experiments is shown.
Figure 1. Schultz et al. 2014

A

Log_{10} PFU/mL

h after infection

SINV
cAP-7
dAP-7

***

B

Log_{10} PFU/mL

h after infection

VEEV
cAP-7
dAP-7

***

C

percent of cells infected

MOI

virus

h

SINV VEEV cAP-7 dAP-7

5 50 5 50 5 50 5 50

8 24

5 50 5 50 5 50 5 50

8 24 48

5 50 5 50 5 50

8 24
Figure 2.  

Schultz et al. 2014
Figure 3. Schulte et al. 2012

![Graph showing percent cell survival over days after infection for different virus strains and MOIs.](http://jvi.asm.org/download)

- cAP-7
- dAP-7
- cAP-7 SINV MOI 5
- dAP-7 SINV MOI 5
- cAP-7 VEEV MOI 50
- dAP-7 VEEV MOI 50
Figure 4. Schultz et al. 2014

Mock | SINV | VEEV
---|---|---
6 | 12 | 24 | 48 | 6 | 12 | 24 | 48 | 6 | 12 | 24 | 48

PE2 E1/E2 C

AP-7

PE2 E1/E2 C

AP-7
Figure 5. Schultz et al. 2014

** Log$\text{_{10}}$ PFU/mL **

h after infection

LACV
cAP-7
dAP-7

***

***

***
Figure 6.

A

B

Schultz et. al. 2014

Schultz et. al. 2014
Figure 8.

Schultz et. al. 2014

A

B

Log_{10} pfu/mL

5  24  5  24  5  24  h

normal differentiation media  dAP-7 sups

Relative Gene Expression

cAP-7, normal  cAP-7 + differentiation media  cAP-7 + differentiation media from dAP-7  dAP-7, normal