Author/s:
Narayanan, D;Moily, N;Mcquilten, HA;Kedzierska, K;Mackenzie, JM;Kedzierski, L;Fazakerley, JK

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Immature Brain Cortical Neurons Have Low Transcriptional Competence to Activate Antiviral Defences and Control RNA Virus Infections

Divya Narayanan[a] Nagaraj Moily[b] Hayley A. McQuilten[a]
Katherine Kedzierska[a] Jason M. Mackenzie[a] Lukasz Kedzierski[a, c]
John K. Fazakerley[a, c]

[a]Department of Microbiology and Immunology at the Peter Doherty Institute of Infection and Immunity, The University of Melbourne, Melbourne, VIC, Australia; [b]Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, VIC, Australia; [c]Faculty of Veterinary and Agricultural Sciences at the Peter Doherty Institute of Infection and Immunity, The University of Melbourne, Melbourne, VIC, Australia

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Alphavirus · Interferon · Neuron · Innate immunity

Abstract
Virus infections of the central nervous system (CNS) cause important diseases of humans and animals. As in other tissues, innate antiviral responses mediated by type I interferons (IFNs) are crucially important in controlling CNS virus infections. The maturity of neuronal populations is an established critical factor determining the outcome of CNS virus infection. Using primary cultures of mouse cortical neurons, we investigated the relationships between neuronal maturation, type I IFN responses, and the outcome of Semliki Forest virus infection. The virus replicated better, infected more cells, and produced higher titres of infectious viruses in immature neurons. Complete transcriptome analysis demonstrated that resting immature neurons have low transcriptional competence to mount antiviral responses. They had no detectable transcription of the genes *Ddx58* and *Ifih1*, which encode key RNA virus cytoplasmic sensors RIG-I and MDA5, and very low expression of genes encoding key regulators of associated signalling pathways. Upon infection, immature neurons failed to mount an antiviral response as evidenced by their failure to produce chemokines, IFNs, and other cytokines. Treatment of immature neurons with exogenous IFNβ prior to infection resulted in antiviral responses and lower levels of virus replication and infectious virus production. In contrast, resting mature neurons generated a robust antiviral response. This was augmented by pretreatment with IFNβ. Infection of mature neurons derived from IFNAR$^{-/-}$ mice did not make an antiviral response and replicated virus to high levels.

Introduction

Central nervous system (CNS) virus infections are a significant global public health burden [1]. Arthropod-borne viruses (arboviruses), mainly the RNA alphaviruses and flaviviruses, are responsible for epidemics with a high burden of neurological disease. Examples include Japanese encephalitis virus (JEV), Zika virus, and the equine encephalitis viruses [2, 3]. In humans, and exper-

Lukasz Kedzierski and John K. Fazakerley contributed equally to this work.

Karger@karger.com
www.karger.com/jin

Correspondence to:
Lukasz Kedzierski, lukaszk@unimelb.edu.au
John K. Fazakerley, john.fazakerley@unimelb.edu.au

imentally in laboratory mice, RNA virus infections of the CNS are generally acute infections, which are rapidly fatal or which rapidly resolve with or without clinical consequence. Arboviral encephalitides are generally more severe in children than in adults. For example, infection of children with JEV results in mortality of a third, recovery with neurological sequelae for another third, and complete recovery without sequelae for the final third [4]. Neurological complications, particularly in the developing embryo and in children, are a serious health issue and include major brain deformities, cognitive impairment, epilepsy, and Parkinsonian conditions. The same phenomenon is observed in experimental animal infections where alphaviruses, flaviviruses, and several other neurotropic viruses, including measles, are more lethal in young than in adult rodents. Experimental studies demonstrate that this age-related virulence is not linked to developmental changes in adaptive immunity but to developmental changes in neural cell functions, including potentially cellular defence and innate immune responses [5].

Neuronal maturation is a complex process. In the developing brain, axonal growth, synapse formation, connectivity, and electrical activity are prerequisites for neuronal survival and are associated with many molecular, biochemical, and neurophysiological changes. In contrast, mature neurons are highly specialized, long-lived, existentially precious cells which generally possess limited regenerative properties, heightened cellular defence mechanisms, and a low propensity to initiate programmed cell death [5].

A growing body of evidence suggests that neuronal cells can actively mount an antiviral response in the CNS. Mature neurons, both differentiated human neuronal cultures and rodent primary neurons, can detect and respond to virus infections [6]. This includes expression of pattern recognition receptors [6, 7] and an enhanced ability to constrain viral replication in response to cytokine stimulation [8, 9]. Differentiated neuronal cell lines in vitro have been shown to express the interferon (IFN) transcriptional regulators IRF-7 and IRF-9 and demonstrate a general increase in responsiveness to exogenous stimulation with type I IFN [10–12]. In vivo, infected mature brain neurons produce type I IFNs [13], have the IFN transcription regulator IRF-3 pathway active [14], and express IFN-stimulated gene (ISG)-12 [15].

Infections of mice with the related alphaviruses, Semliki Forest virus (SFV), and Sindbis virus (SINV) have been extensively studied as models to understand virus neuropathogenesis and neuroimmunology [16]. In the current study, we investigated IFN responses to SFV infection in immature and mature mouse primary cortical neurons. Immature neurons replicated virus to high levels more rapidly than mature neurons. In response to infection, immature neurons did not produce type I IFNs (α or β), the chemokines RANTES (CCL5), IP-10 (CXCL10), KC (CXCL1), or MCP-1 (CCL2), or the proinflammatory cytokine interleukin-6 (IL-6), whereas infected mature neurons produced all these inflammatory modulators. In contrast, in both immature and mature neurons, pretreatment with IFNβ prior to SFV infection upregulated most of the above modulators and reduced virus replication. Transcriptomic profiling of cellular defence and immune related genes showed highly distinct profiles in immature and mature neurons, both when virus-infected or when pretreated with IFNβ and then infected. While many genes were upregulated upon infection of mature neurons, fewer and different genes were upregulated upon infection in immature neurons. Pretreatment with IFNβ prior to infection also resulted in different patterns of gene expression in immature and mature neurons.

Materials and Methods

Viruses and Infection

SFV4 with a Firefly luciferase reporter gene (SFV4 FFLuc) inserted between duplicated nsP2 cleavage sites at the nsP3/4 junction as a cleavable reporter [17] was used for infections (kindly provided by Andres Merits, University of Tartu, Estonia). Neurons were infected at a multiplicity of infection (MOI) of 1 at 37°C for 1 h. The infectious inoculum was removed, and cells were washed with media. Prior to infection, cultures were pretreated or mock-treated with type I IFN. Mouse IFNβ (PBL Interferon Source) was resuspended in 0.1% (wt/vol) BSA in PBS and neurons were pretreated with 100 U/mL for 24 h prior to infection. Viral titres in neuronal cultures infected with SFV were determined by plaque forming unit assay on Vero cells as previously described [18] or 50% tissue culture infectious dose on BHK-21 cells [19].

Primary Mouse Cortical Neuron Culture

Seventeen- to eighteen-day-old embryos (E17-E18) of C57BL/6 mice were dissected in cold Hank's Balanced Salt Solution (HBSS) with 0.3% bovine serum albumin (HBSS+). Cortices were resuspended in HBSS+ with 10× Tryple Select (ThermoFisher) and DNase I (10 U/mL), incubated for 20 min at 37°C, and then centrifuged at 500 *g* for 5 min at RT. The pellet was resuspended in HBSS+ supplemented with DNase I (10 U/mL), $MgSO_4$ (150 mM), and trypsin inhibitor (Sigma Aldrich), triturated, and allowed to stand for 30 s for the debris to settle. The aqueous portion was collected and centrifuged at 500 *g* for 5 min at RT. The pellet was resuspended in neural basal media (Life Technologies) supplemented with 1% B27 (Life Technologies), 200 mM GlutaMAX (Life Technologies), and 1% penicillin/streptomycin solution. Neurons were seeded into 12-well plates coated with 0.1 μg/μL poly-D lysine hydrobromide (Sigma Aldrich) and 10 ng/μL mouse laminin (Life Technologies). Cells were grown at 37°C, and culture media were

replenished every 3 days. Immature and mature neurons were grown for 3 and 12 days, respectively. Immunostaining using a Neural 3-colour Immunocytochemistry Kit (R&D Systems) for β-tubulin, O4, and GFAP demonstrated the cultures to be neuronal with only occasional glial cells. Neuronal cell morphology was used as an indicator of neuron maturity [20]. Increased numbers and branching of neurites occurred between days 3 and 12. Cell viability was assessed using CellTitre-Blue Cell Viability Assay (Promega).

Luciferase Assay

Firefly luciferase assay was used as a surrogate measure of virus replication [21]. Immature and mature neurons were either mock-treated or pretreated with 100 U/mL IFNβ for 24 h and infected with SFV4-FFluc at MOI = 1. Samples were collected 4, 12, and 24 h post-infection. Cells were lysed with 100 μL passive lysis buffer (Promega) supplemented with 1× protease inhibitor (Roche) (30 min, 4°C with rocking). Firefly luciferase activity was measured using 20 μL of lysate in a CLARIOstar plate reader (BMG LABTECH) according to manufacturer's instructions (Promega). Samples were analysed in triplicate.

Cytokine and Chemokine Multiplex Assay

Cytokines and chemokines were analysed using LEGENDplex Mouse Anti-Virus Response Panel (BioLegend) which allows quantification of 13 mouse cytokines and chemokines, including IFNγ, CXCL1, TNFα, CCL2, IL-12p70, CCL5, IL1β, CXCL10, GM-CSF, IL-10, IFNβ, IFNα, and IL-6. The assay was performed as per manufacturer's instructions. Samples were analysed using BD FACSCANTO II flow cytometer and data were analysed using the LEGENDplex data analysis software (BioLegend).

Intracellular Staining and FACS Analysis

FACS analysis was performed in order to determine the proportion of neurons infected with SFV and the mean fluorescence intensity (MFI) determined by staining the cells for SFV nsP3 or nsP1 protein. Briefly, infected neurons (in triplicates) were washed with PBS and gently dissociated from wells using Tryple Select (ThermoFisher), then centrifuged at 500 *g* for 5 min at 4°C. Cells were transferred to 96 U-bottom well plates (ThermoFisher) and cell viability performed using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher) for 10 min at RT in the dark. Cells were washed with FACS buffer (0.5% BSA, 1 mM EDTA in PBS) and permeabilized with Cytofix/Cytoperm™ fixation/permeabilization solution (BD Biosciences) as per manufacturer's instructions. Staining for SFV proteins was done using polyclonal rabbit anti-nsP3 or anti-nsP1 primary antibody for 1 h on ice. Cells were washed twice with BD wash buffer and stained with anti-rabbit IgG AF488 conjugated secondary antibody (kind gift from Prof. Danny Hatters, University of Melbourne) for 15 min at RT. The cells were resuspended in FACS buffer and analysed on BD FACSCANTO II. Data were analysed using FlowJo software.

RNA Sequencing

RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Whole cell RNA used for further processing had RNA integrity values >9. Library preparation was done with Illumina HiSeq 2500 RNA Tru-Seq Library Kit for polyA-enriched mRNA and sample quality was checked using Agilent Tape station 2200 for cDNA quality post-library preparation. Single-end sequencing was done using a NextSeq high throughput sequencer on a single flow cell to obtain ~400 M reads. The Illumina HiSeq 2500 platform was used to generate single-end 100 bp.

RNA Sequencing Data Analysis

FastQC files were checked for quality using the FastQC reporter (version 0.11.9) [22] and checked for Phred scores. Per base sequencing quality was >30 (i.e., less than 1 in 1,000 chance of a wrong base call). There were no warnings for overrepresented sequences, duplicate sequences, GC content, or ambiguous base call numbers in any of the samples. Reads were mapped to the reference mouse transcriptome (Mus_musculus.GRCm38.78.gtf) using the Salmon ultrafast aligner (version 1.3.0) [23]. Mapped reads were quantitated to the gene-level estimates (gene count data) and imported to R statistical tool (version 4.0.3) using the tximport package (version 1.18.0) [24] and differential expression analysis was carried out using the limma-voom package (version 3.46.0) [25] in Bioconductor (version 3.11). Gene ontology (GO) and pathway enrichment analysis was conducted using the EGSEA package (version 3.13) [26] in Bioconductor and goana in the limma package (version 3.48.3). Further canonical pathway activation and upstream regulator predictive analysis was performed using Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., USA). Sample and gene clustering was performed using the pheatmap package (version 1.0.12) and Venn diagrams generated using VennDiagram (version 1.6.20).

Statistical Analyses

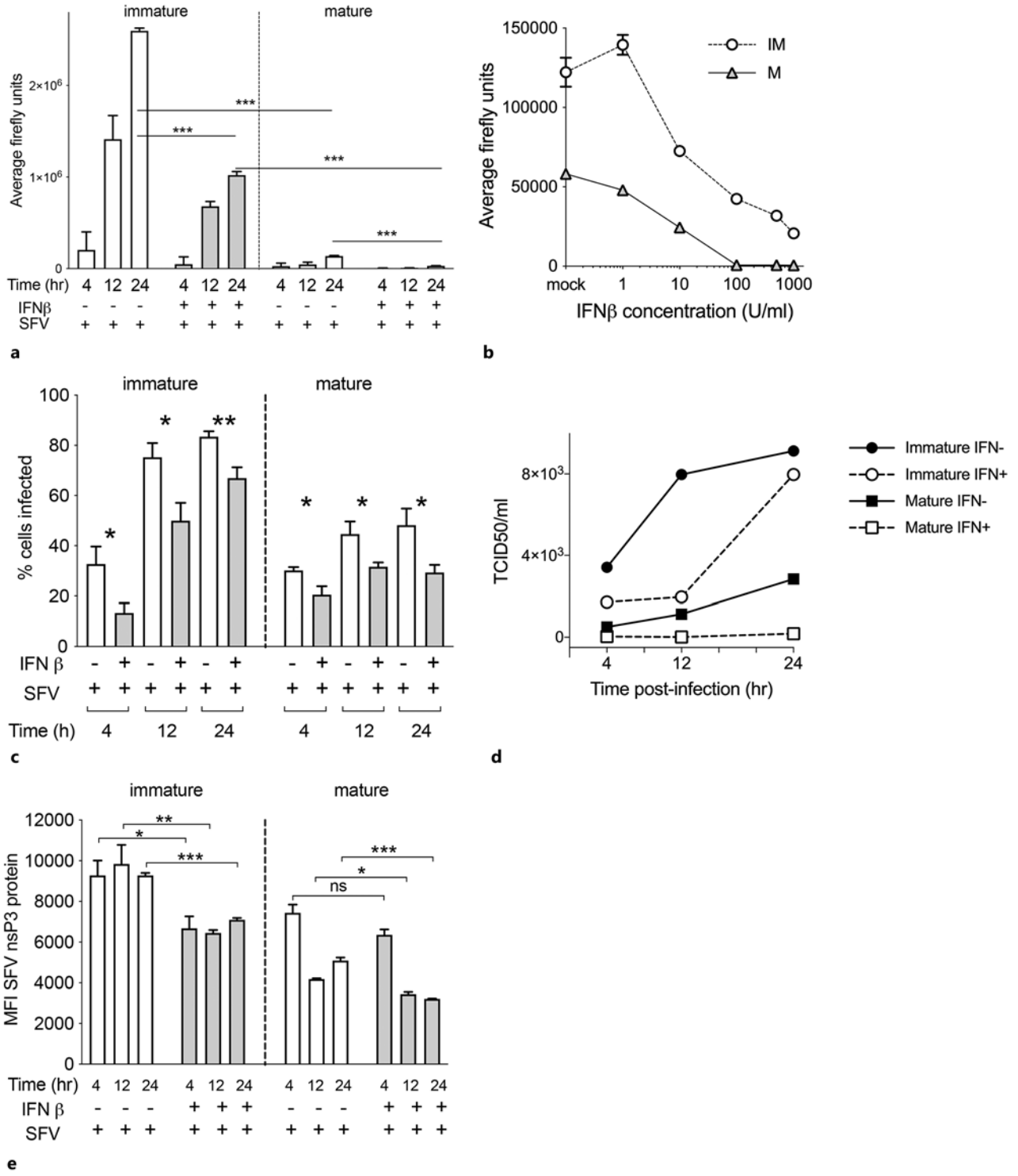
Statistical analyses were performed using the Student's unpaired *t* test provided within GraphPad Prism 9 software.

Results

The model system used in these studies were mouse primary brain cortical neurons derived from 17- to 18-day-old embryos and cultured in vitro for 3 or 12 days. We refer to the former as immature and the latter as mature neurons. Consistent with this, the mature neurons had many more processes and interconnections than the immature cells. Infections were carried out using SFV carrying well-characterized molecular marker, firefly luciferase. This virus was based on the SFV4 strain and has been described in our previous publication [27].

Immature Cortical Neurons Respond to IFNβ but Cannot Effectively Control Virus Replication

We compared the response of immature and mature primary mouse cortical neurons to SFV infection, with and without pretreatment with IFNβ. SFV with a firefly luciferase marker expressed from the virus replicase was used to measure virus replication. Immature neurons replicated virus to significantly higher levels than mature neurons (Fig. 1a). In a dose-dependent manner, treatment with IFNβ 24 h prior to SFV infection reduced virus



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replication in both immature and mature neurons 24 h post-infection (Fig. 1a, b). We conclude that SFV replicates better in immature than mature neurons and that both cell types are able to respond to IFNβ and reduce virus replication.

To determine whether these differences in virus replication resulted from changes in the levels of replication in infected neurons or from the number of neurons infected, the percentage of cells infected and the MFI of intracellular nsP3 in the infected cells were measured by flow cytometry over the first 24 h of infection (Fig. 1c, e). In the absence of pretreatment with IFNβ, at 4 h post-infection, the percentage of infected cells was approximately the same for both immature and mature neurons, 32% and 30%, respectively (Fig. 1c). The percentage of infected immature neurons had risen to almost 80% by 12 h and to 83% by 24 h. In contrast, the percentage of mature neurons infected remained at less than 50% at both of these time points. Pretreatment with IFNβ significantly reduced the percentage of infected cells in both cultures (Fig. 1c) with a greater proportional reduction at 4 h in immature than mature neuronal cultures. Although immature neuronal cultures showed a significantly lower rate of infection following IFNβ pretreatment than following mock-pretreatment, the percentage infected cells in the culture clearly increased between 4 h (13%) and 24 h (67%). In contrast, the percentage of infected cells in the mature neuronal cultures following pretreatment with IFNβ did not increase much with time (20% at 4 h vs. 29% at 24 h). For immature neuronal cultures, either mock-pretreated or pretreated with IFNβ, the mean levels of nsP3 in individual cells for each treatment group were not significantly different at 4, 12, and 24 h. However, at each time point, the mean levels of nsP3 were less in the IFNβ pretreated group (Fig. 1e). In mature neuronal cultures, the mean intracellular levels of nsP3 were noticeably lower at 4 h post-infection and were significantly reduced at 12 and 24 h. There were also clear differences in production of infectious virus from mature and immature cultures and the ability of IFNβ pretreatment to reduce this differed between cultures (Fig. 1d).

Collectively, these data demonstrate that SFV replicates better in immature than mature neurons. This is apparent from the luciferase analysis of virus replication, the percentage of infected cells in the cultures, the mean cellular levels of virus nsP3, and the production of infectious virus. The data also clearly demonstrate that both immature and mature neurons respond to exogenous type I IFNβ pretreatment and that in each case, IFN pretreatment resulted in lower levels of virus replication and production. However, only mature, differentiated neurons had the ability to maintain an IFN-induced antiviral state, resulting, over the first 24 h of infection, in only minimal increases in the percentage of infected cells in the culture, a reduction in mean levels of nsP3 in these infected cells, and no increase in infectious virus production.

Absence of Type I IFN Signalling Ablates Mature Neurons Resistance to SFV Infection

To directly determine the role of the type-I IFN response in mouse cortical neurons, we prepared immature and mature neuronal cultures from mice with a genetic deletion of the type-I IFN receptor (IFNAR1$^{-/-}$ mice). In both neuronal differentiation states, SFV infection led to strong virus replication, widespread infection, and high infectious virus production. As expected, and in contrast to wild-type (WT) cells (Fig. 1), this could not be rescued by pretreatment with IFNβ (Fig. 2). There was no significant difference in the levels of luciferase activity produced by immature and mature neurons infected with SFV4-FFLuc irrespective of whether they were pretreated with IFNβ or not (Fig. 2a). In the wt neurons, firefly luciferase levels increased in all experimental groups from 4 to 12 to 24 h; whereas in the IFNAR1$^{-/-}$ neurons, luciferase levels were not significantly different across these times and infection in these cultures was associated with widespread cell death by 24 h. There was also no significant difference

Fig. 1. The dynamics of SFV infection differ between immature and mature neuronal cultures but both respond to exogenous IFNβ. **a** Levels of FFLuc activity in primary cortical neuron cultures at different time points following infection (MOI 1) with SFV4-FFLuc and pretreatment with IFNβ (100 U/mL). Bars represent the mean of triplicates and error bars represent SEM. A representative of 3 independent experiments is shown. **b** Dose-response curve showing the effect of IFNβ on SFV replication at 24 h pos-tinfection in immature (circles) and mature (triangles) neurons. **c** Flow cytometry analysis of the percentage of infected cells (positive for nsP3 staining) in immature and mature neurons at different time points following SFV4-FFLuc infection. Bars represent the mean of 7–8 replicates from 2 independent experiments; error bars represent SEM. **d** Titres of infectious virus in culture supernatants. Each time point represents a mean of 2–3 replicate culture supernatants. **e** FACS analysis of MFI of nsP3 protein determined by immunostaining. Each bar represents a mean of 3 replicates, error bars represent SEM. A representative of 2 independent experiments is shown (all panels: *$p < 0.05$, **<0.005, ***<0.001).

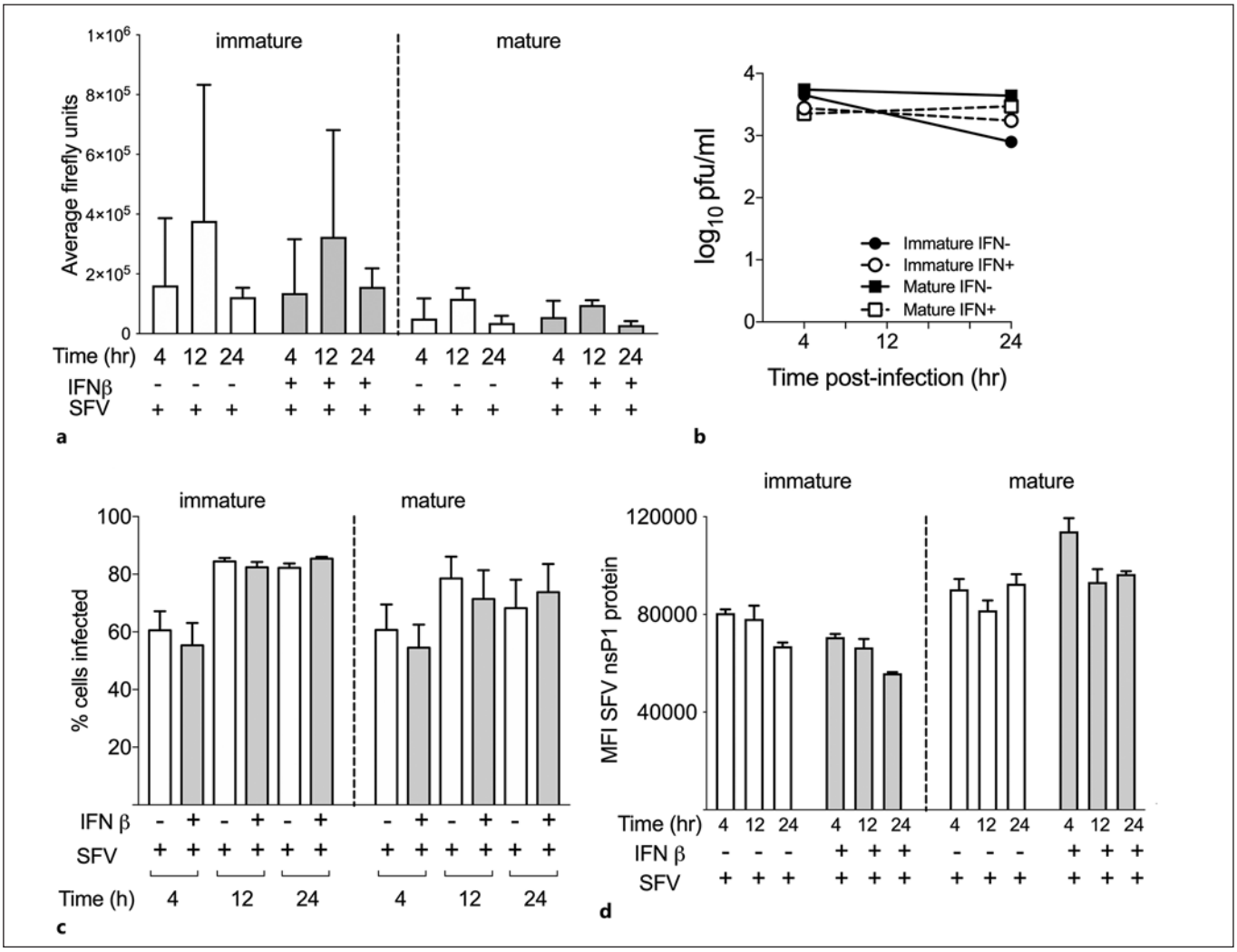

Fig. 2. Type I IFN signalling mediates the relative resistance of mature neurons to SFV infection. **a** Levels of FFLuc activity in IFNAR$^{-/-}$ primary cortical neurons at different time points following infection with SFV4-FFLuc. Bars represent the mean value (6–15 replicates) and error bars represent SEM. Combined data from 2 independent experiments are plotted for 4 and 12 h time points, data for 24 h time point come from one experiment. Cells were pretreated with IFNβ (100 U/mL). **b** Titres of infectious virus in culture supernatant from IFNAR$^{-/-}$ neurons. Each time point is the mean of supernatants from two replicate cultures. **c** Flow cytometry analysis of the percentage of infected cells (cells positive for nsP1) in IFNAR$^{-/-}$ immature and mature neuronal cultures at different time points following SFV4-FFLuc infection. Bars represent the mean of 7–11 replicates from 2 independent experiments, error bars represent SEM. **d** Mean levels of virus (nsP1) protein in IFNAR$^{-/-}$ neurons following infection with SFV4-FFLuc determined by FACS analysis of MFI of nsP1 protein immunostaining. Each bar represents a mean of 3–4 replicates, error bars represent SEM. A representative of 2 independent experiments is shown.

in the IFNAR1$^{-/-}$ neurons between the experimental groups, immature or mature neurons, with or without IFN pretreatment, in the amount of infectious virus released to the culture supernatant (Fig. 2b), the percentage of cells infected (Fig. 2c), or the MFI values in nsP1^{+} cells (Fig. 2d). We conclude that removal of type I IFN signalling in mature neurons reduces their resistance to infection rendering them virtually indistinguishable from immature neurons. Clearly, relative to immature neurons, the IFN response provides some protection to SFV infection for mature neurons. Given that both neuronal differentiation states can respond to exogenous IFN, a likely explanation of their differential response of infection is that the immature neurons do not produce IFN in response to SFV infection.

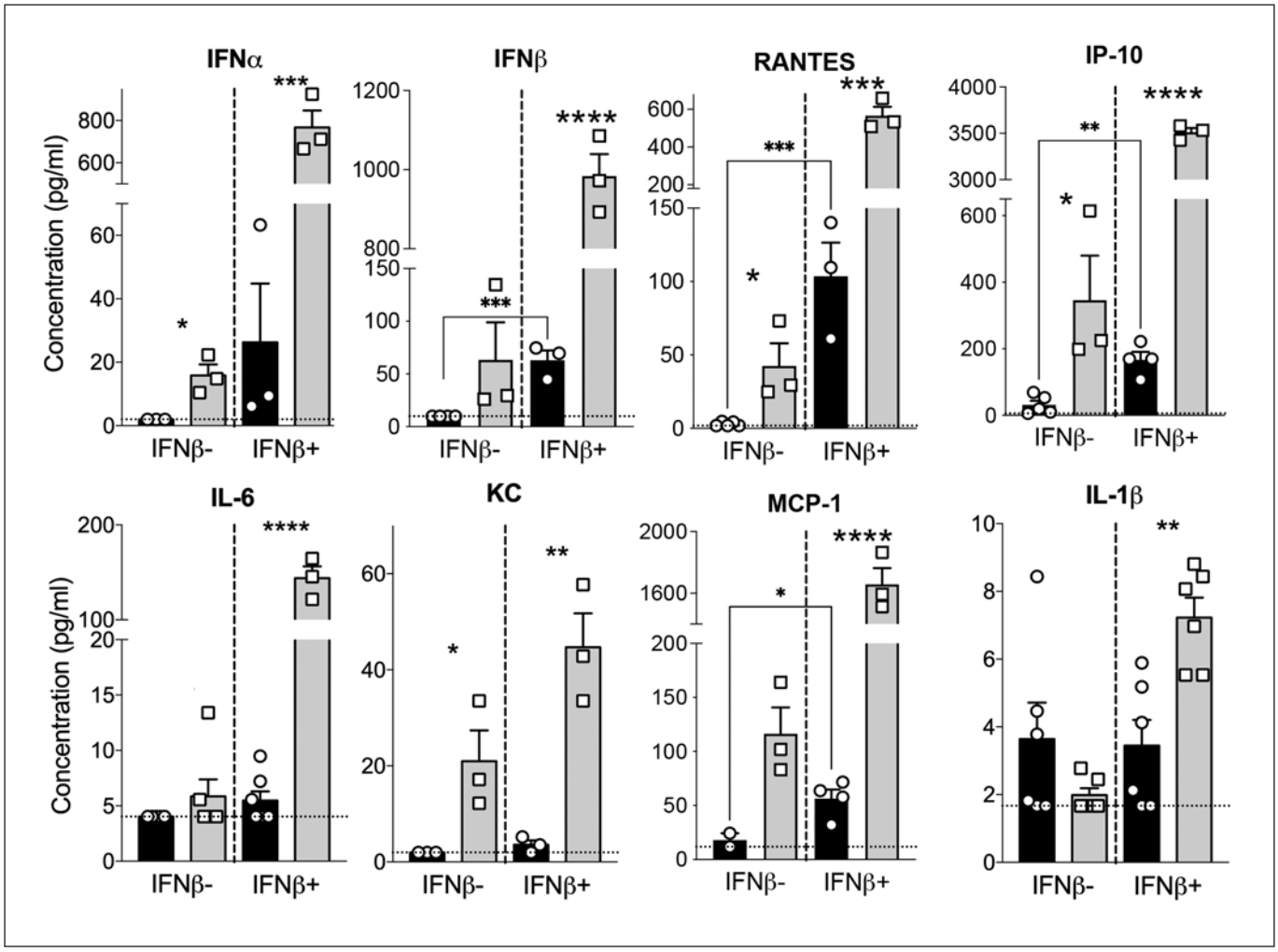

Fig. 3. Immature neurons do not make type I IFN. Multiplex analysis of cytokine and chemokine levels in culture supernatant from immature (black bars) and mature (grey bars) neurons in the absence or presence of IFNβ pretreatment. Each bar represents a mean of 3–4 replicates, error bars represent SEM. The dashed lines represent the detection limits of each assay. *$p < 0.05$, **<0.005, ***<0.001, ****<0.0001.

Immature Cortical Neurons Have Restricted Ability to Produce Antiviral Cytokines following SFV Infection

To compare antiviral factors produced by the two neuronal cultures, a multiplex analysis of cytokines and chemokines in culture supernatants was undertaken. Neurons were infected with SFV4-FFLuc at MOI = 1 and supernatants analysed 24 h post-infection. Following SFV infection, immature neurons did not make detectable levels of IFNα or IFNβ proteins (Fig. 3); whereas, both type I IFNs were clearly detected in culture supernatants from infected mature neuronal cultures. Furthermore, levels of CCL5 (RANTES), CXCL-10 (IP-10), IL-6, CXCL-1 (KC), and CCL-2 (MCP-1) were consistently below the detection limits of the assay in infected immature neuronal cultures but were all detectable in infected mature neuron cultures. IFNγ, GM-CSF, TNFα, IL-10, and IL-12 were below the detection level in all culture supernatants (data not shown). In mature neurons, pretreatment with IFNβ, 24 h prior to infection, strongly augmented production of IFNβ, IFNα, IL-6, RANTES, IP-10, MCP-1, KC, and IL-1β; for IFNα, IFNβ, RANTES, IP-10, IL-6, and MCP-1, this was at least a 10-fold increase. For immature neurons, pretreatment with IFNβ 24 h prior to infection resulted in low levels of all the above modulators with levels of IFNβ, RANTES, IP-10, and MCP-1 significantly higher than in the mock-treated immature neurons.

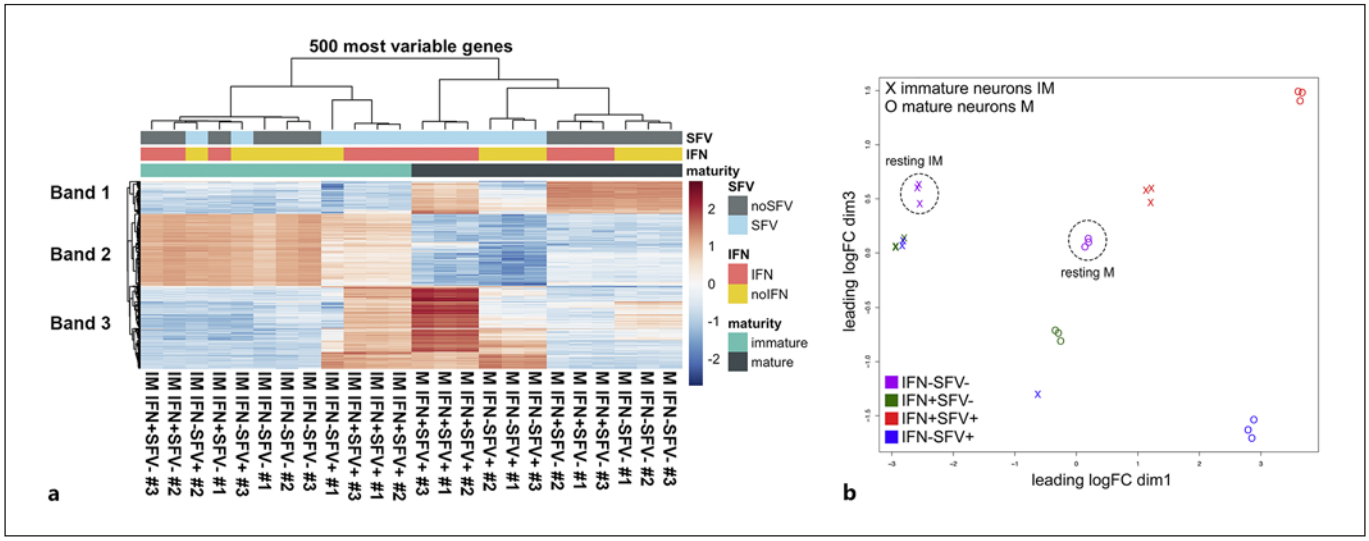

Fig. 4. Transcriptomic analysis shows changes in gene expression related to neuronal maturation, SFV infection, and IFN pretreatment. **a** Scaled (*Z* score) expression of the 500 most variable genes in all samples represented in a heatmap with unsupervised clustering of both samples and genes. **b** Multidimensional scaling plot showing variation in the expression of the top 1,000 most variable genes. Dimension 1 (axis *X*) separates immature from mature neurons, while variation between samples is seen in dimension 3 (axis *Y*). The leading $\log_2$FC (base 2 logarithm of fold change) is the average of the largest absolute $\log_2$FC between each sample. The following groups were analysed: resting neurons (IFN−SFV−), neurons primed for 24 h with 100 U/mL IFNβ (IFN+SFV−) or infected with SFV4 (IFN−SFV+), and neurons primed with IFNβ and infected with SFV4 (IFN+SFV+). Both immature and mature neurons were either pretreated with IFNβ or mock pretreated, and cells were infected for 1 h with SFV4 or mock-infected. All cells were harvested 12 h post-infection.

These data indicate that immature neurons do not mount a robust antiviral response regardless of prior treatment with type I IFN, although priming with IFNβ does enhance production of selected cytokines and chemokines. In contrast, mature neurons are able to produce type I IFNs and other antiviral cytokines in response to SFV infection and this process is greatly augmented by priming with IFNβ.

Transcriptional Profiling of Neurons following SFV Infection

To further investigate the differences between immature and mature neurons, we performed genome wide transcriptional profiling of primary cortical immature or mature neurons either mock- or IFNβ-treated for 24 h and then infected or mock-infected with SFV. The libraries were analysed by RNA-seq with three technical replicates of each sequenced using the HiSeq2500 platform. As expected, there were major differences in transcriptomic profile between immature and mature neurons in their response to alphavirus infection and IFNβ pretreatment (Fig. 4). Analysis of the 500 most variable genes revealed three major bands of genes (Fig. 4a). Bands 1 and 2 are broadly associated with maturity of neurons irrespective of the treatment (online suppl. Table S1; see www.karger.com/doi/10.1159/000525291 for all online suppl. material). Band 3 are genes upregulated in response to SFV infection. For immature neurons, this only occurs if the cells are treated with IFNβ prior to infection. For mature neurons, the response is considerably stronger if the cells are pretreated with IFNβ. Band 3 contains genes known for their antiviral function such as oligoadenylate synthase (*Oas*) gene family, *Irf7*, *Ifih1*, or *Ifn* gene family (online suppl. Table S1).

Multidimensional scale analysis of variance in the expression of the top 1,000 genes revealed the same clustering of the replicates and clear separation of the experimental groups (Fig. 4b). One replicate of the immature, non-IFNβ pretreated, SFV-infected group was an outlier. This is also visible in the data in Figure 4a. Interestingly, immature neurons pretreated with type I IFN and mock-infected or infected without prior IFN clustered close to the basal (resting) control samples, indicating limited changes in gene expression under either condition. This was also the case for mature neurons treated with IFNβ. Whereas, immature neurons pretreated with IFN and

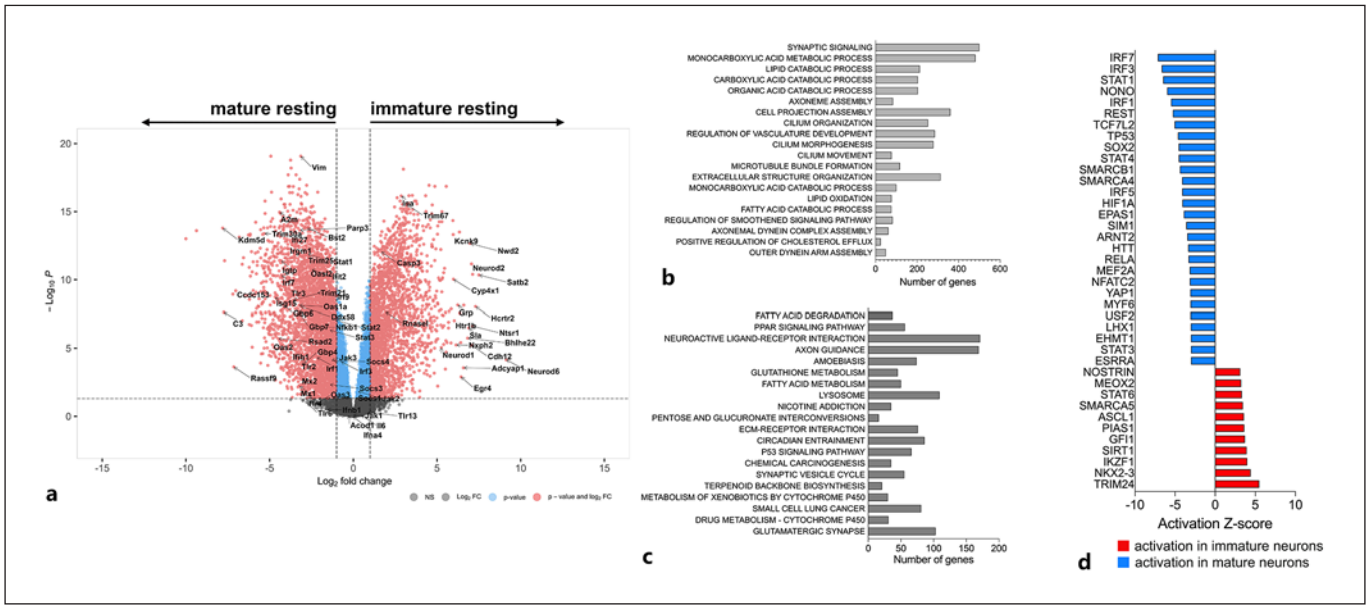

Fig. 5. Comparison of gene expression profiles of resting immature and mature neurons. Gene expression was analysed 12 h post-mock-infection. **a** Volcano plot showing the relative gene expression distribution of all (14,933 variables) differentially expressed genes comparing resting immature to resting mature neurons. The horizontal line corresponds to a false discovery rate – corrected significance value of <0.05. The vertical lines denote the minimal fold-change for the most-differentially-expressed genes. Differentially expressed genes with p value <0.05 are coloured blue, genes with p value <0.05 with absolute $\log_2$ fold change of 1 or above are coloured red, while non-significant genes are coloured black. GO (**b**) and KEGG (**c**) pathway enrichment analysis of genes differentially expressed between resting immature and mature neurons. The 20 most significantly enriched GO terms for biological processes and KEGG pathways in resting immature neurons compared to resting mature neurons are shown. Significantly enriched ($p < 0.05$) GO terms and KEGG pathways ranked by significance are shown with the number of differentially expressed genes identified in each gene set. **d** IPA of gene expression data showing upstream transcriptional regulators predicted to differ between resting immature and mature neurons. The x-axis depicts the activation Z-score. The top transcription regulators predicted to be more highly activated (Z-score >3; $p < 0.05$) in immature, relative to mature neurons, are depicted on the right (red) while those predicted to be more highly activated (Z-score <−3; $p < 0.05$) in mature, relative to immature neurons, are depicted on the left (blue). KEGG, Kyoto Encyclopedia of Genes and Genomes.

then infected, as well as mature neurons pretreated or not with IFN and then infected, underwent more dynamic changes in gene expression.

Based on the above analysis, we hypothesized that immature and mature neurons differentially express genes at the basal level, which affect responsiveness to IFN and to SFV infection. Inherent gene expression variations between resting immature and mature neurons are shown in Figure 5. The expression (FDR < 0.05) of 5,184 genes was significantly downregulated and of 4,299 genes was significantly upregulated in resting immature neurons compared to resting mature neurons. There was no significant difference in expression for 5,480 genes.

The top genes upregulated in resting immature neurons (right-hand side of the volcano plot, Fig. 5a) include neuronal differentiation family (Neurod) genes (*Neurod1*, *Neurod 2*, *Neurod 6*), internexin neuronal intermediate filament protein alpha (*Ina*), tripartite motif-containing protein 67 (*Trim67*), hypocretin receptor 2 (*Hcrtr2*), neurotensin receptor 1 (*Ntrs1*), cadherin 12 (*Cdh12*), Src-like adaptor (*Sla*), basic helix-loop-helix family member e22 (*Bhlhe22*), ribonuclease L (*RNaseL*), and Caspase 3 (*Casp3*). These are all known to be involved in regulating neuronal functions.

In resting mature neurons, relative to immature neurons (left-hand side of volcano plot, FDR < 0.05, Fig. 5a), significantly higher basal expression was observed for multiple genes with known antiviral functions. These include: *Kdm5d*, complement *C3*, *Trim30a*, *Ddx58* (RIG-I), *Ifih1* (MDA5), Toll receptors (*Tlr2*, *Tlr3*, *Tlr4*), transcription factors implicated in IFN induction and signalling (*NfkB*, *Irf3*, *Irf7*, *Irf9*, *Stat2*, *Stat3*), and IFN inducible genes implicated in antiviral responses (*Rsad2*, *Bst2*, *Oas1a*, *Oasl2*, *Isg15*, *Mx1*, *Mx2*, *Ifit2*, *Igtp*, *Ifi27*).

These results show that the basal gene expression of immature and mature primary cortical neurons differs significantly and that the mature neuron transcriptome is enriched in immune defence molecules. GO (Fig. 5b) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Fig. 5c) pathway analyses revealed enrichment of synaptic signalling, metabolic processes gene sets, neuroactive ligand-receptor interactions, and nonimmune pathways in resting immature neurons. Ingenuity Pathway Analysis (IPA) of the gene expression data indicated that transcriptional regulators involved in immune response such as IRF7 (*Z*-score: −7.15), IRF3 (−6.67), STAT1 (−6.48), IRF1 (−5.5), STAT4 (−4.54), IRF5 (−4.11), and STAT3 (−3.01) were, relative to mature neurons, conspicuously inhibited in immature resting neurons. Conversely, transcription factors involved in neuronal development and function such as TRIM24 (*Z*-score: 5.48), NKX2-3 (4.41), SIRT1 (3.92), GFI1 (3.709), and ASCL1 (3.57) were activated in resting immature neurons compared to resting mature neurons (Fig. 5d). In resting immature neurons, predicted transcription regulators are involved in neuronal commitment and differentiation rather than innate immune pathways.

Comparison of gene expression in virus-infected immature and mature neurons showed dramatic differences (Fig. 6a, b). In immature neurons, while some genes relevant to an antiviral response appeared to change, few reached significance (Fig. 6a). Of the genes with a significant change, 17 were downregulated and one, *Mphosph9*, was upregulated. Expression values for *IFNb* and *IFNa4*, while higher in infected than control cells, were not significantly different. The genes significantly downregulated upon infection compared to their basal expression levels in immature neurons included genes implicated in antiviral responses (*Bst2, Oasl2, Oas1a, Irf7, Ifi27, Trim30a*) and components of the major histocompatibility complex class I antigen presentation pathway (*B2m*). In contrast, following SFV infection, mature neurons (Fig. 6b) displayed a robust response to infection with large numbers of significantly upregulated (4,204) and downregulated (4,391) genes. Amongst the highest levels of upregulation were *IFNb*, *IFNa4*, and *IL6*, characteristic of an early antiviral response. Other upregulated genes included PRR receptors – *Ifih1*, *Ddx58*, TLR family members (*Tlr6*, *Tlr2*, *Tlr13*); transcription factors – *NFκB*, *Stat2*, *Irf1*; ISGs – *Oas2*, *Oas3*, *Mx1*, *Mx2*, *Isg15*, *Rsad2*; and IFN pathway regulators like *Socs1* and *Socs3*.

We identified key transcription regulators predicted to be activated based on the individual differential gene expression profiles following SFV infection (Fig. 6c–e). The upstream regulator predictive analysis showed that transcriptional regulators IRF3, IRF7, and STAT1 were inhibited and TRIM24 was activated in immature SFV-infected neurons compared to resting cells; whereas for others, we did not detect pathway activation (Fig. 6c). In mature SFV-infected neurons compared to resting controls, we identified a group of activated transcriptional regulators related to antiviral response and IFN pathway. These included STAT1, RELA, IRF7, CREB1, IRF3, IRF1, and NFKB1 (Fig. 6d). Figure 6e compares transcriptional pathway regulation between infected immature neurons and mature infected neurons and shows that immature neurons do not initiate protective responses.

Transcriptional Profiling of Neurons following IFNβ Priming and SFV Infection

The difference in responses to infection of immature and mature neurons was blunted when they were pretreated with IFNβ as both cell types now responded by increasing gene expression. Compared to resting immature neurons, immature neurons pretreated with IFNβ and infected with SFV showed significant downregulation of 3,777 genes and significant upregulation of 4,233 genes with 6,923 genes showing no significant change (Fig. 7a). Compared to resting mature neurons, mature neurons pretreated with IFNβ and infected with SFV showed significant downregulation of 5,130 genes and significant upregulation of 3,348 genes with 6,365 genes showing no significant change (Fig. 7b). Direct comparison of IFNβ pretreated and infected neurons showed that in immature neurons, relative to mature neurons, 4,922 genes were significantly reduced in expression and 5,301 genes were significantly increased, with 4,710 genes showing no change in expression (Fig. 7c). Compared to resting neurons, or indeed SFV-infected neurons, following priming with IFNβ and subsequent SFV infection, both immature and mature neurons had significantly higher levels of expression of genes involved in virus sensing and antiviral responses. These included *Ddx58* (RIG-I, a virus RNA sensor); *Ifih1* (MDA5, a virus RNA sensor); *Irf7* and *Irf9* (IFN regulatory transcription factors controlling IFN-inducible genes); *Ifi27* (a regulator of IFN-induced apoptosis); *Oas* (an inhibitor of RNA synthesis); and ISGs such as *Isg15* (a ubiquitin-like protein), *Rsad2* (viperin), and *Mx1/Mx2* (antagonists of virus replication). Immature neurons had lower levels of all these genes relative to mature neurons (Fig. 7c). There were also numerous genes that had higher expression in immature than mature neurons. For the most part, these were not known antiviral genes with the exception of *Rnasel*.

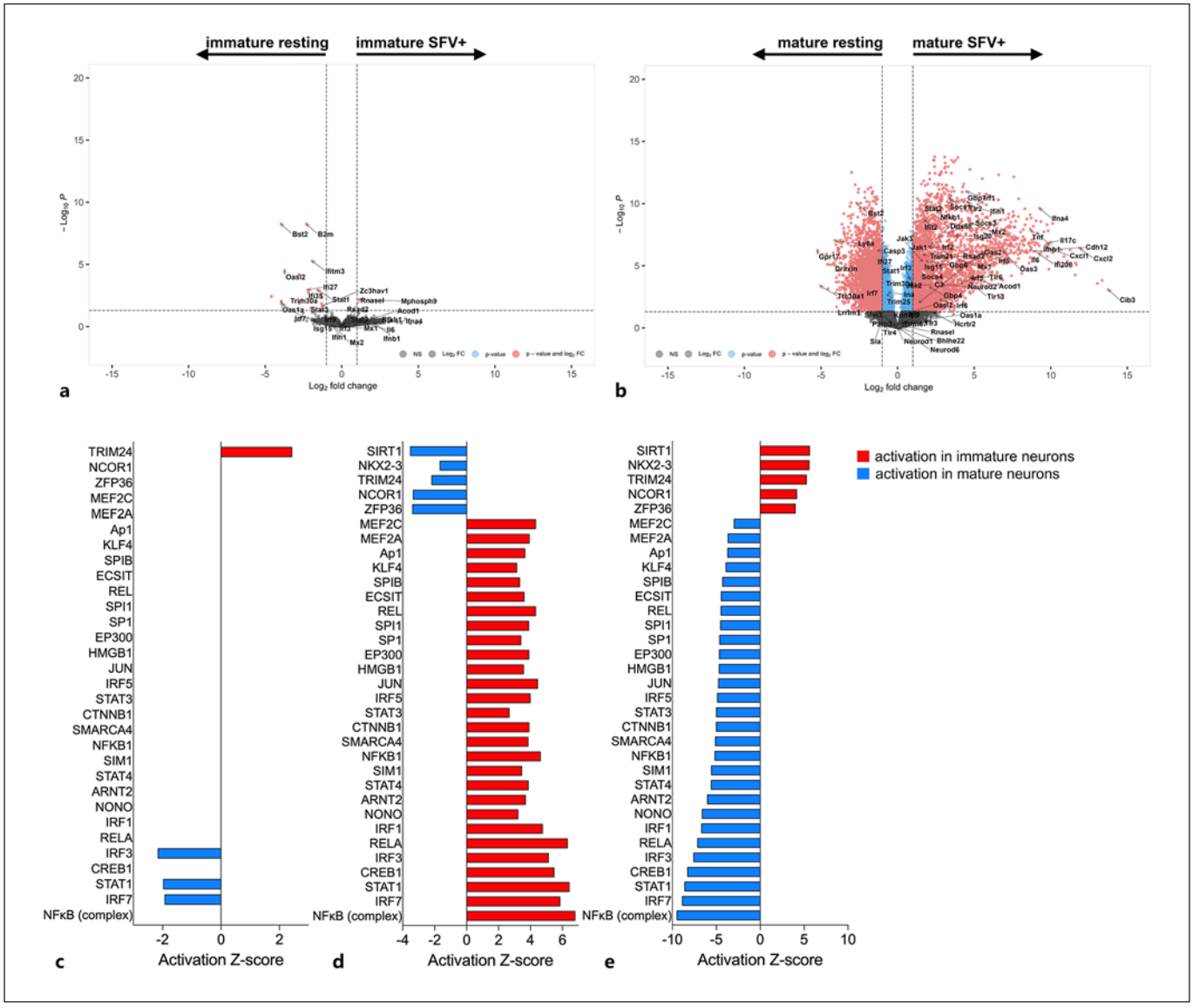

Fig. 6. Comparison of differential gene expression profiles of SFV-infected immature and mature neurons. Gene expression was analysed 12 h post-infection (SFV) or mock-infection. Volcano plots showing relative gene expression distribution of differentially expressed genes in SFV-infected compared to resting immature neurons (**a**) and relative gene expression distribution of differentially expressed genes in SFV-infected compared to resting mature neurons (**b**). The horizontal line corresponds to a false discovery rate – corrected significance value of <0.05. The vertical lines denote the minimal fold-change for the most differentially expressed genes. Differentially expressed genes with p value <0.05 are coloured blue, genes with p value <0.05 with absolute $\log_2$ fold change of 1 or above are coloured red, while nonsignificant genes are coloured black. IPA of gene expression data showing upstream transcriptional regulators predicted to differ between SFV-infected and resting immature neurons (**c**), SFV-infected and resting mature neurons (**d**), and SFV infected immature and mature neurons (**e**). The x-axis depicts the activation Z-score. The top transcription regulators with Z-score >3; $p < 0.05$ are depicted in red, while those with Z-score <−3; $p < 0.05$ are depicted in blue.

More detailed analysis of SFV-induced changes in the neuronal transcriptome linked to the IFN signalling pathway identified significant differences in gene clusters related to the IFN response (Fig. 7d). Five clusters composed of 7,902 differentially expressed genes of interest were identified and linked to biological process GO terms (Fig. 7e). Cluster 1 contains genes broadly downregulated during SFV infection relative to the resting state, in both

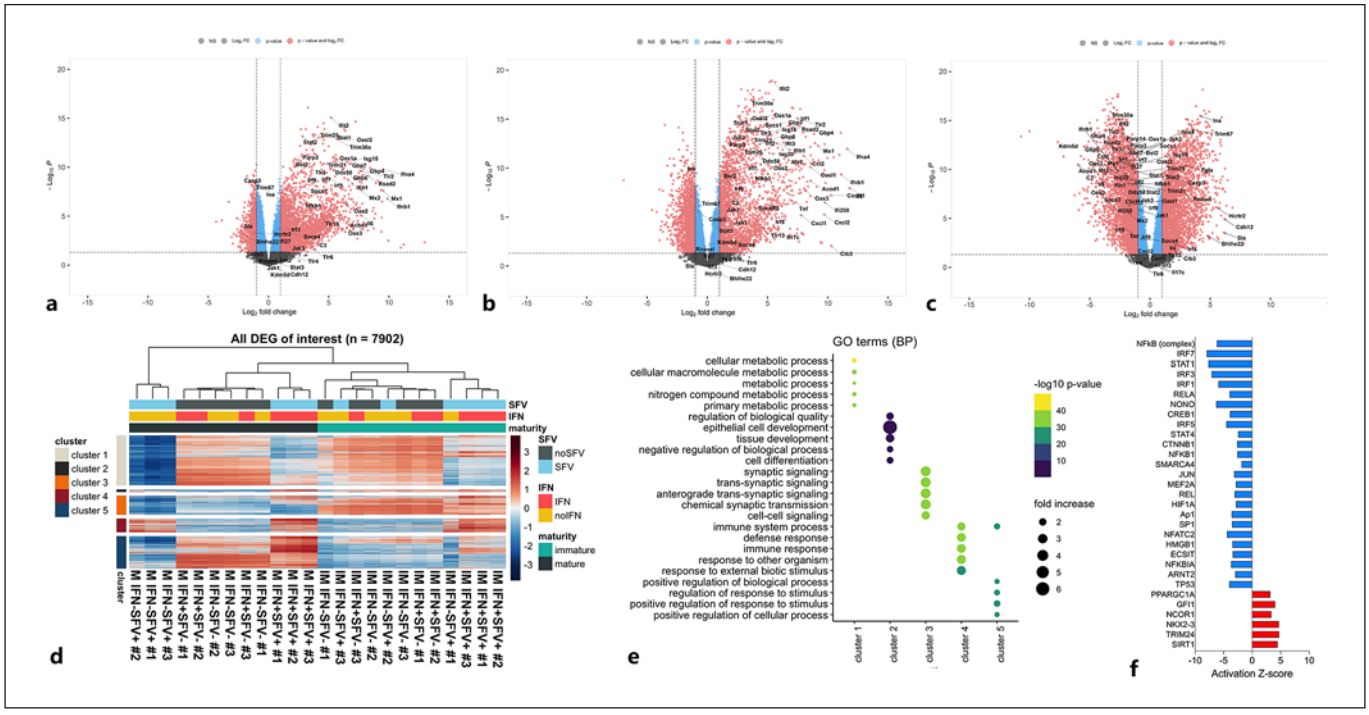

Fig. 7. Analysis of differential gene expression profiles of immature and mature neurons following pretreatment with IFNβ and SFV infection. Gene expression was analysed 12 h post-infection (SFV) or mock-infection and 36 h post-IFN treatment (100 U/mL) or mock-treatment. Volcano plots showing relative gene expression in immature neurons pretreated with IFNβ and infected with SFV compared to resting immature neurons (**a**); relative gene expression in mature neurons pretreated with IFNβ and infected with SFV compared to resting mature neurons (**b**); and relative gene expression in immature neurons pretreated with IFNβ and infected with SFV compared to mature neurons pretreated with IFNβ and infected with SFV (**c**). Differentially expressed genes with p value <0.05 are coloured red, genes with >2 absolute log 2-fold change and p value <0.05 are coloured blue, while nonsignificant genes are coloured black. **d** Unsupervised clustering of datasets by genes differentially expressed relative to resting neurons. Genes clustered by expression (Z-score), with clusters labelled on side panel. **e** The top 5 enriched GO terms for biological processes in each of the clusters identified in **d**. Over-represented GO terms were ranked by p value and five terms with the lowest p values in each cluster were selected. Enrichment was adjusted for overall abundance of each GO term in the genome, with the size of symbols in (**e**) representing N genes$_{(GO\ term)}$ in cluster/N genes in cluster ÷ N genes$_{(GO\ term)}$ in all expressed genes/N genes in all expressed genes. **f** IPA of gene expression data showing upstream transcriptional regulators predicted to differ between IFN+SFV+ immature and IFN+SFV+ mature neurons. The x-axis depicts the activation Z-score. The top transcription regulators predicted to be more highly activated (Z-score >3; $p < 0.05$) in IFN+SFV+ immature, relative to IFN+SFV+ mature neurons, are depicted on the right (red), while those predicted to be more highly activated (Z-score <−3; $p < 0.05$) in IFN+SFV+ mature, relative to IFN+SFV+ immature neurons, are depicted on the left (blue).

mature and immature neurons. These include many genes involved in metabolic processes. Small cluster 2 is more polarized in mature than immature neurons, and members of this cluster are involved in developmental processes. Cluster 3 is linked to differences in cellular maturity and genes in this cluster have higher expression in immature neurons, under all conditions. These genes are involved in synaptic and trans-synaptic signalling and appear to be linked to the early developmental stage of neurons. Cluster 4 consists of genes upregulated in SFV infected mature neurons, with or without IFNβ pretreatment and in infected immature neurons if they are IFNβ pretreated. Genes in this cluster fall into immune defence and immune response categories (GO:0002376, GO:0006952, GO:0006955). Cluster 5 is characterized by genes with higher expression in mature neurons, either at rest or following IFNβ treatment. Interestingly, genes in this cluster are downregulated upon SFV infection of mature neurons, but this process is reversed by IFNβ pretreatment. In general, cluster 5 genes are downregulated in immature neurons regardless of experimental conditions. This cluster is enriched in genes belonging to the GO terms immune system process (GO:0002376), and broadly to positive regulation of biological processes

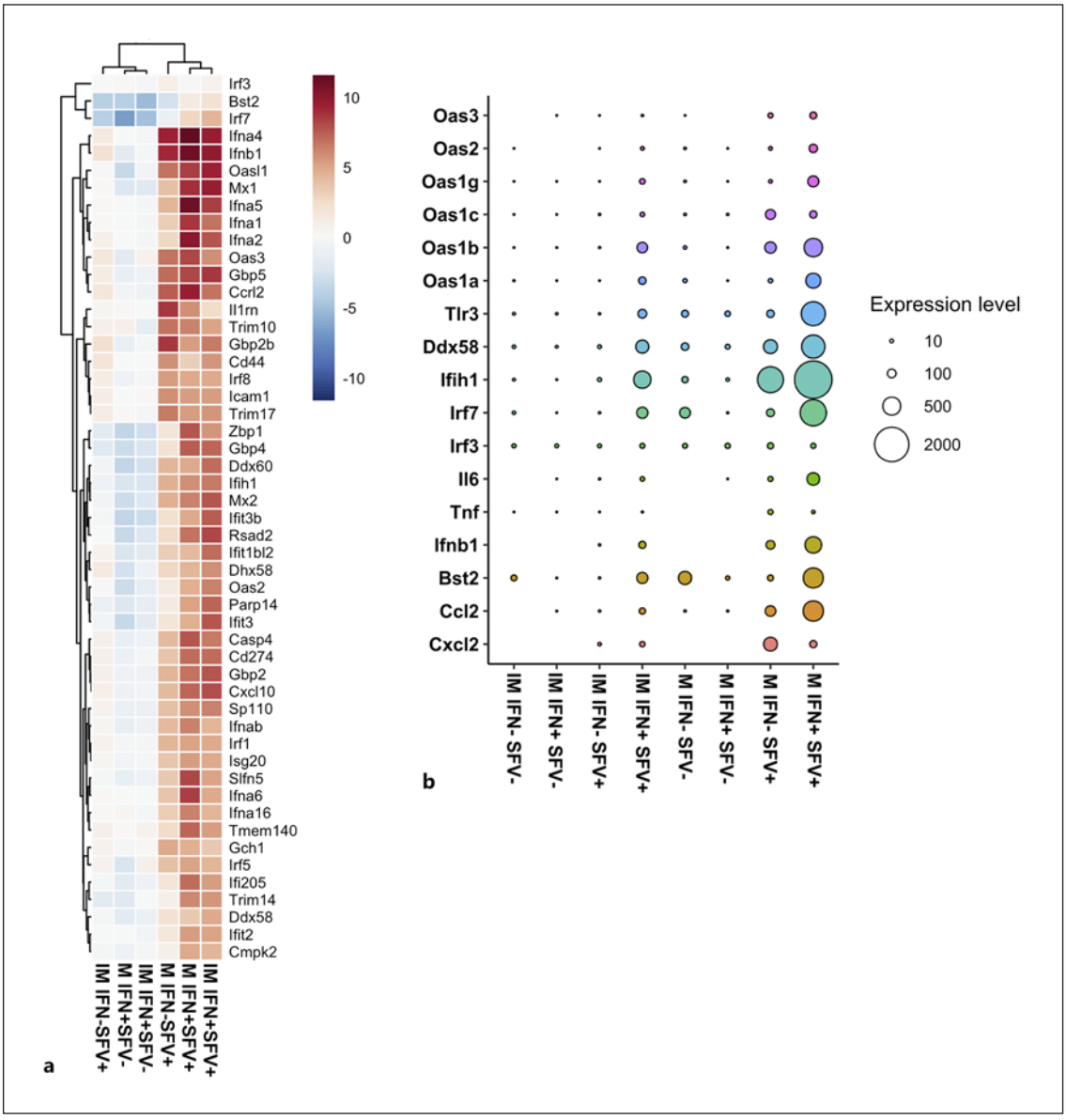

Fig. 8. Analysis of expression of genes relevant to type I IFN pathway and antiviral responses. Expression was analysed 12 h post-infection (SFV) or mock-infection and 36 h post-IFN treatment (100 U/mL) or mock-treatment. **a** Heatmap arranged by unsupervised clustering depicting $\log_2$FC of selected antiviral genes; in each case, the experimental condition is relative to resting cells. Genes selected are derived from MSigDB C2 and Reactome Interferon alpha/beta signalling and ISG sets [28]. **b** Expression profiles of selected ISGs showing levels of expression in resting neurons, following IFNβ pretreatment, SFV infection, or both. The size of the bubble depicts the gene expression level (counts per million) and colours denote different genes.

(GO:0048518, GO:0048522) and responses to stimuli (GO:0048584, GO:0048583).

Pathway activation and upstream regulator predictive analyses revealed a similar activation profile following IFNβ pretreatment and SFV infection in both immature and mature neurons when compared to their resting state (online suppl. Fig. S1). This included activation of cellular defence and innate immune pathways regulated by transcription factors including NFKB1, RELA, STAT4, and CREB1. Comparison between IFNβ-treated, SFV-infected, immature and mature neurons demonstrated that cellular defence and innate immune pathways regulated by transcription factors including STAT1, IRF3, IRF1, IRF5, and CREB1 were more strongly activated in mature than

immature neurons (Fig. 7f). These data indicate that similar mechanisms are in play in response to IFNβ pretreatment and SFV infection in mature and immature neurons with the key difference being the magnitude of the response.

Analysis of fold change (Fig. 8a) and expression profiling (Fig. 8b) of selected genes relevant to IFN signalling and antiviral responses revealed that in immature neurons, expression of these genes was upregulated when cells were treated with IFNβ and then infected with SFV. These genes were not upregulated in immature neurons when they were treated with IFNβ alone, or when they were only infected. In contrast, for mature neurons, these genes were upregulated by SFV infection alone, and this was augmented by prior treatment with IFNβ. IFNβ alone did not induce their expression.

Collectively, these data indicate that murine immature primary cortical neurons failed to activate, or at least to strongly activate, gene expression of innate defence pathways following either SFV infection or stimulation with exogenous type I IFN. They did, however, respond to SFV infection when pretreated with IFNβ. Conversely, mature neurons developed a robust antiviral response following SFV infection alone. Treatment with exogenous IFNβ had no obvious effect on gene expression but strongly augmented the response to subsequent SFV infection.

Discussion

In the studies reported here, we used mouse primary immature and mature cortical neurons to determine any differences in the responses of these cells to infection with SFV. The virus replicated better in cultures of immature neurons than in cultures of mature neurons with higher levels of virus protein, a greater percentage of cells infected, and higher production of infectious virus. Mature neurons, but not immature neurons, developed a clear and strong antiviral response to infection as determined by changes in gene expression; activation of innate immunity pathways; and production of chemokines, IFNs, and other cytokines. Neither immature nor mature neurons showed clear changes in antiviral gene expression in response to treatment with IFNβ, but IFNβ treatment prior to SFV infection did prime both neuronal differentiation states to mount a more robust antiviral response on subsequent infection. Thus, immature neurons failed to mount an antiviral response upon infection but could do so if primed with IFNβ, whereas mature neurons mounted a good antiviral response upon infection. This was ablated in neurons devoid of type I IFN receptors and augmented by prior treatment with IFNβ.

In these studies, neuronal cultures were treated with IFNβ 24 h prior to infection. Given that immature neurons were unable to produce additional IFN upon infection (Fig. 3, 6, 8), the antiviral effect of exogenous IFNβ pretreatment would have been transient. This is consistent with the reduction in infectious virus titres in IFN pretreated immature neuronal cultures, relative to controls, at 4 and 12 h post-infection which had disappeared by 24 h, where titres were essentially no different (Fig. 1e). This recovery of virus production was not observed in IFN treated and infected mature neurons which make their own IFNs in response to SFV infection.

Immature neurons derived from either WT or IFNAR$^{-/-}$ mice were equally susceptible to SFV infection, suggesting that type I IFN signalling does not play a major role in antiviral defences of immature neurons (Fig. 2). Upon infection, WT immature neurons did not upregulate antiviral genes (Fig. 6, 8) and were unable to produce cytokines or chemokines (Fig. 3). In response to IFNβ treatment alone, immature neurons did not detectably increase antiviral gene expression, but IFN pretreatment did prime these cells, indicating their responsiveness to IFN, as determined by their increased antiviral gene expression, cytokine and chemokine production, and reduced levels of virus replication and infectious virus production, in response to subsequent infection.

Upon infection, mature neurons upregulated many antiviral genes including IFNs and several ISGs (Fig. 6–8); these included *Mx*, *2′–5′ Oas* and *Trim* family genes, *Rsad2* (viperin) and *ISG15* [29–33]. These cells also produced other cytokines and chemokines (Fig. 3) and replicated virus, notably to lower levels than immature neurons (Fig. 1). Following IFNβ pretreatment and infection, expression of antiviral genes and production of cytokines and chemokines were further upregulated in mature neurons and virus replication and infectious virus production further downregulated (Fig. 1, 3, 8). Relative to WT cells, infection of mature neurons derived from IFNAR$^{-/-}$ mice demonstrated higher levels of virus replication and infectious virus production. However, the levels of virus replication (Fig. 2a), while higher than in WT mature neurons, remained lower than in immature neurons, suggesting that while the IFN response has a major role in reducing virus replication in mature neurons, there are also other factors related to neuronal differentiation which curtail virus replication in mature neurons.

A striking difference between neuronal differentiation states was a significantly lower level of expression of both *Ddx58* (RIG-I) and *Ifih1* (MDA5) ($\log_2$FC –3.16 and

−3.26, respectively) in resting immature versus resting mature neurons. This is consistent with previous reports that RIG-I and MDA5 are not expressed, or expressed at only very low levels, in resting 5–7-day-old cortical neurons in vitro [14, 34]. Both of these molecules are key sensors of RNA virus infections and inducers of IFN responses [35–38]. For SFV, in mouse embryonic fibroblasts, both molecules are involved in type I IFN induction and subsequent restriction of viral replication [39]. The importance of RIG-I in response to neuronal virus infection has also been observed with JEV, where knockdown of RIG-I in mature neurons resulted in reduced production of proinflammatory cytokines and chemokines and increased virus replication [40]. Neither *Ddx58* nor *Ifih1* were significantly upregulated in the datasets for either neuronal phenotype by IFNβ pretreatment alone (Fig. 8b). However, as discussed above, exogenous IFNβ effects appear to wane over time and by the time of cell harvest, 36 h post-IFN treatment, any gene expression induced by exogenous IFN may have returned to normal. Transient production of RIG-I or MDA5 in IFN pretreated immature neurons, or transient increased production in IFN pretreated mature neurons, could underlie the stronger response to infection that followed IFNβ priming. Neither of these two virus sensor genes were significantly upregulated 12 h post-SFV infection in immature neurons.

The IFN signalling pathway activated by RIG-I and MDA5 includes IRF1, IRF3, and NFκB [41]. Infected mature neurons showed significantly increased gene expression of these three regulators along with other key transcription factors related to activation of innate immune system pathways including IRF5, IRF7, STAT1, STAT3, STAT4, JUN, and RELA. IPA indicated that these pathways were active in infected mature neurons (Fig. 6, 7). However, these transcription factors were not present, or present at only very low levels, in resting or infected immature neurons and these innate immune pathways were not activated by infection in immature cell cultures (Fig. 5–8). We conclude that immature neurons are not transcriptionally competent to mount innate antiviral responses as they lack both the sensors of infection and the key signalling molecules.

In conclusion, our studies demonstrate that immature primary mouse cortical neurons in culture neither have the key cytoplasmic sensors RIG-I and MDA5 to detect virus nor do they have the key transcriptional regulators to signal activation of these sensors. In consequence, upon infection, these cells do not activate antiviral signalling pathways, produce chemokines, IFNs, ISGs, or other cytokines. This unresponsiveness results from a low level of transcriptional competence. This is not changed by virus infection, but it is overcome by exogenous prior administration of IFN, indicating that immature neurons can respond to IFN, even though they do not produce it upon infection. In contrast, resting mature neurons do transcribe genes for virus detection, including *Ddx58* (RIG-I) and *Ifih1* (MDA5), and genes that regulate key innate immunity responses and they respond to virus infection through production of IFNs, ISGs, chemokines, and cytokines. This response is ablated in mature neurons with no functional IFN system and augmented by prior treatment with IFNβ.

The inability of immature cortical neurons to sense and respond to virus infections due to their low level of transcriptional competence to mount antiviral defence responses, including most notably an IFN response, provides an explanation as to why many CNS virus infections are more devastating in the developing than in the developed brain. However, while this is likely to be a major factor governing the age-related pathogenesis of neurotropic virus infections, IFN responses are not the only factor because mature neurons without a functional IFN system, while replicating virus to higher levels than those with IFN competence, did not replicate virus to the even higher levels seen in immature neurones.

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Statement of Ethics

Animal experiments were conducted according to the Prevention of Cruelty to Animals Act 1986, the Prevention of Cruelty to Animals Regulations 2008, and the National Health and Medical Research Council (2013) Australian Code for the Care and Use of Animals for Scientific Purposes. Ethics was approved by the University of Melbourne Animal Ethics Experimentation Committee (1714184).

Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Author Contributions

Divya Narayanan: investigation, methodology, data analysis, data visualization, and writing – review and editing. Nagaraj Moily: data curation, data analysis, data visualization, and writing – review and editing. Hayley A. McQuilten: data analysis, data visualization, and writing – review and editing. Katherine Kedzierska: resources, supervision, and writing – review and editing. Jason M. Mackenzie: conceptualization, supervision, and writing – review and editing. Lukasz Kedzierski: conceptualization, supervision, investigation, data visualization, and writing – original draft preparation, review, and editing. John K. Fazakerley: conceptualization, funding acquisition, supervision, and writing – review and editing.

Data Availability Statement

The data that support the findings of this study will be openly available from the NCBI Gene Expression Omnibus Repository database (https://www.ncbi.nlm.nih.gov/geo/) upon manuscript publication under accession number GSE195828.

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