Innate STAT1-Dependent Genomic Response of Neurons to the Antiviral Cytokine Alpha Interferon

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Alpha/beta interferons (IFNs-α/β) are cytokines that play an essential role in the host defense against viral infection. Our previous studies have shown that the key IFN signaling molecule STAT1 is highly elevated and activated in central nervous system neurons during viral infection and in transgenic mice with astrocyte production of IFN-α (glial fibrillary acidic protein [GFAP]-IFN-α), suggesting that neurons are a very responsive target cell population for IFNs. To elucidate the genomic response of neurons to IFN-α, we undertook studies both in vitro and in vivo. Gene chip analysis was applied to RNA from IFN-α-treated or untreated primary cortical neuronal cultures derived from embryonic day 15 fetal wild-type or STAT1 knockout (KO) mice. The expression of 51 known and 5 unknown genes was increased significantly by more than twofold after exposure of wild-type but not STAT1 KO neurons to IFN-α. Some more highly expressed genes included IFN-induced 15-kDa protein, ubiquitin-specific protease 18, glucocorticoid attenuated response genes, IFN-induced GTPases, and the chemokine CXCL10. For several of these genes, the gene chip findings were confirmed by RNase protection assays. In addition, examination of the expression of some of these selected genes revealed that they were increased in neurons in the brain of either GFAP-IFN-α mice or mice infected with lymphocytic choriomeningitis virus. In conclusion, our study revealed a robust STAT1-dependent genomic response of neurons to IFN-α, highlighting an innate potential of these cells to defend against viral infection in the brain.

Alpha/beta interferons (IFNs-α/β) were among the first cytokines to be discovered and characterized. Initially identified as antiviral proteins, IFNs-α/β are now known to be pleiotropic and plurifunctional molecules with antiproliferative and immune-regulatory properties (32, 41), which has led to their widespread therapeutic use in patients with certain viral infections (16), various cancers (21), and autoimmune disorders (11, 38). The binding of IFNs to their common receptor, named the IFN-α/β receptor (IFNAR), activates a signaling cascade involving the Janus kinase /signal transducer and activator of transcription (STAT) pathway (41). Specifically, IFNAR activation triggers the downstream Janus kinase-mediated tyrosine phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 molecules form heterodimers that translocate to the nucleus and associate with another transcription factor, interferon regulatory factor 9. This trimolecular complex is termed interferon-stimulated gene factor 3 (ISGF3). Finally, ISGF3 binds to a specific DNA recognition site termed the interferon-stimulated response element to modulate the transcription of a large number of genes that contribute to the functional biology of these cytokines.

The role of IFNs-α/β in protection against viral infection was demonstrated initially in mice treated with neutralizing antibodies against these cytokines (18) and has since been further confirmed in IFNAR-deficient mice, which develop lethal infection when inoculated with virus that is normally nonlethal in wild-type counterparts (29). In the central nervous system (CNS), the importance of IFN-α in homeostasis against viral infection is underscored by studies showing increased or decreased sensitivity to the infection of neurotropic viruses in mice with IFN-α/β receptor deficiency (15, 26) or in mice with CNS-targeted expression of the IFN-α gene, respectively (1, 9, 40). Interestingly, in comparison to the periphery, for many viruses, infection of the CNS is associated with reduced tissue destruction and decreased symptoms, despite the continued presence of viral expression (30). These observations suggest that neurons could be equipped with the means to combat the replication and spread of otherwise cytopathic viruses in the CNS, providing protection for the host against potentially catastrophic neurologic disease that would otherwise result from injury or loss of these largely nonrenewable cells.

One little-studied but potentially effective host strategy for limiting viral infection in the CNS could be local production of antiviral mediators such as IFNs-α/β. For example, intrathecal synthesis of IFN-α occurs in patients with CNS viral infections (14). Cells intrinsic to the brain produce IFN-α/β including astrocytes and microglia and possibly neurons (44). Similar to astrocytes, cultured neurons respond to treatment with IFNs with increased 2′5′-oligoadenylate cyclase (24) and major histocompatibility complex class I (MHC-I) gene expression (27). Furthermore, in examining the IFN-α signaling cascade, we found that a key transcriptional factor mediating IFN-α signaling, STAT1, was highly increased and activated in neurons in the brain of glial fibrillary acidic protein (GFAP)-IFN-α transgenic mice (43).

The observation that neurons have elevated levels of STAT1

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in the brain of GFAP-IFN-α transgenic mice led us to propose that these cells were a sensitive and highly responsive target for the IFNs-α/β (7, 43). This, in turn, suggested that neurons might have an intrinsic capacity to mount an antiviral response after exposure to IFN-α. At present, however, knowledge of the extent of the neuronal response to IFN-α remains quite limited. With the advent of DNA microarray technology, global gene profiling is now possible and offers a powerful new tool to study the transcriptional responses of cells to biological modifiers such as the IFNs-α/β (2). Therefore, in the present study we examined the genomic response of primary cultured neurons prepared from embryonic day 15 (E15) mouse brain to IFN-α using Affymetrix DNA microarray gene chip analysis.

MATERIALS AND METHODS

Mice and infection with LCMV. STAT1 knockout (KO) mice (10) (129/Sv/Ev background) were kindly provided by Robert D. Schreiber (Washington University School of Medicine, St. Louis, MO), and wild-type mice of the same background strain were purchased from Taconic (Germantown, NY). The genotypes of mice were verified by PCR analysis of tail DNA. Two lines of GFAP-IFN-α transgenic mice, GIFN12-low expressor and GIFN39-moderate expressor, were generated and characterized as described previously (7, 8). Mice (12 weeks old) were killed; the brains were removed and fixed or snap-frozen for further analysis as described below. For lymphocytic choriomeningitis virus (LCMV) infection, 10- to 12-week-old wild-type mice were inoculated intracranially with 25 μl of serum-free Dulbecco's modified Eagle's medium containing 250 PFU of LCMV strain Armstrong 53b as previously described (8). Control mice were injected with vehicle only. Three and 6 days after inoculation, mice were euthanized, and the brain was collected for RNA extraction and histological examination. Handling of mice and experimental procedures were conducted in accordance with the National Institutes of Health (NIH) guidelines for animal care and use.

Primary neuronal culture. Primary cultures of neurons derived from the cerebral cortex and subcortical structures (basal ganglia and hippocampus) were prepared as described previously (45). In brief, brain was removed from E15 mice (wild-type or STAT1 KO), dissected free of meninges, digested at 37°C for 10 min in Hank's balanced salt solution with 0.25% trypsin without Ca^{++} and Mg^{++} (Invitrogen, San Diego, CA). The digestion mixture was then treated with 0.004% DNase (Roche Diagnostics, Indianapolis, IN) and 0.03% soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO). After trituration with a fire-polished Pasteur pipette and washing with HBSS, the single-cell suspension was resuspended in Neurobasal medium with 2% B-27 supplement (Invitrogen), 1% fetal bovine serum (Invitrogen), 10 μM forskolin (Sigma), 45 μM 3-isobutyl-1-methylxanthine (Sigma), and 25 μM glutamate (Tocris Cookson, Ballwin, MO); cells were counted by trypan blue exclusion analysis and plated on a T75 culture flask (Corning, Corning, NY) at a density of 9.0×10^5 cells/ml. After a 48-h incubation (in 5% CO_2 at 37°C), the culture medium was replaced with the same medium but with no serum or glutamate. Neuronal purity of the cultures was ~90 to 95% assessed as described previously (45). Ten days after initiation, the cultures were treated either with murine IFN-α (250 IU/ml; Roche Diagnostics Corp.) or vehicle for 4 h at 37°C and 5% CO_2.

DNA microarray assay and data analysis. Total RNA extracted from the neuronal cultures was prepared using TRIZOL reagent (Life Technologies, Gaithersburg, MD) and further purified using an RNeasy spin column (QIAGEN Inc., Valencia, CA). Standard GeneChip protocols provided by Affymetrix were used for cRNA preparation, sample labeling, hybridization, staining, and scanning procedures as previously described (10, 12). In brief, 5 μg of total RNA from each sample was converted into double-stranded cDNA using a cDNA synthesis kit (InVitrogen, San Diego, CA) with an oligo(dT)$_{24}$ primer containing a T7 RNA polymerase promoter site (Genset, La Jolla, CA). Biotin-labeled cRNA was then generated from the cDNA sample by an in vitro transcription reaction supplemented with biotin-11-CTP and biotin-16-UTP (ENZO, Farmingdale, NY). After purification on an RNeasy spin column (QIAGEN), 20 μg of labeled cRNA was fragmented by mild alkaline treatment at 94°C for 35 min in 40 mM Tris-acetate buffer (pH 8.1) containing 100 mM KOAc and 30 mM MgOAc. Subsequently, labeled cRNA (15 μg) was hybridized to Affymetrix murine U74Av2 chips (Santa Clara, CA) according to the manufacturer's instructions. After washing with sodium chloride-sodium phosphate-EDTA buffer and staining with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR),

the chips were read using an Affymetrix GeneChip scanner, and the data were captured using gene expression software provided by Affymetrix.

All user-definable parameters were set at the Affymetrix default values with identical cutoff values to describe the confidence that a given transcript was detected (present), not detected (absent), or marginal. The details of this algorithm are available at the Affymetrix web site (http://www.affymetrix.com/products /statistical_algorithm_reference_guide.html). For each experiment, four samples (one each from four individual groups) were assayed with U74Av2 chips, and the data were analyzed in the following way. First, the raw data from each of four groups (wild-type control, wild-type plus IFN-α, STAT1 KO control, and STAT1 KO plus IFN-α) were incorporated into a single Microsoft Excel spreadsheet, and the data generated from the probe sets that showed an absolute call of absent in all four chips were excluded. In the calculation of the factor of change between two groups among the different groups, a value of 10 was adopted for those average differences valued less than 10. Then, only those with more than a twofold change (increase or decrease) between the control and IFN-α-treated groups were used for further analysis. Finally, the data were combined from three independent experiments ($n = 3$), and those genes with changes (increase or decrease) twofold or greater were subjected to statistical analysis with a Student's t test to ascertain significance at a P value of <0.05.

RPA. Poly(A)$^+$ RNA was isolated from freshly dissected snap-frozen brain using oligo(dT) (Ambion, Austin, TX) as described previously (4). RNase protection assays (RPAs) were performed, and RNA levels were quantified from autoradiographs by densitometry using NIH Image software (version 1.31) as described previously (23). RPA probes for ISG15, USP18, and GBP3 were synthesized by reverse transcription-PCR, cloned, and verified by sequencing analysis as previously described (23). The targeted sequences for ISG15, USP18, and GBP3 were residues 181 to 381 (GenBank NM_015783), 901 to 1181 (GenBank NM_011909), and 1488 to 1658 (GenBank NM_018734), respectively. Other multiprobe sets for the different STATs and IFN-regulated genes were as described previously (4, 23).

In situ hybridization. Brains were removed and fixed overnight in ice-cold 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). For in situ hybridization, paraffin-embedded sagittal sections (8 μ) were prepared. ^{33}P-labeled cRNA probe transcribed from the linearized ISG15 RPA plasmid described above was used for in situ hybridization performed as described previously (4, 23). Slides were coated with NTB-2 emulsion (Kodak, Rochester, NY) and stored in sealed light-proof boxes at 4°C until development. Prior to mounting, sections were counterstained with Mayer's hematoxylin and dehydrated in graded ethanols.

Statistical analysis. All data are given as the means ± standard error of the means. Data were analyzed by a two-tailed unpaired Student's t test with a significance level at 0.05 or less.

RESULTS

IFN-α-stimulated gene expression in primary cultured neurons. After 2 to 3 days in culture, neurons began to establish contact with each other by extending numerous processes. A dense network of processes was prominent after 10 days in culture, at which time there was no morphological difference visible between the cultures prepared from wild-type and STAT1 null mice (data not shown). To investigate the effects of IFN-α on these neurons, DNA microarray analysis was performed on RNA prepared 4 h after treatment of the cultures with IFN-α or no treatment. Of 12,488 elements on the U75Av2 chip, a total of 56 were increased significantly (P value of <0.05) by twofold or more (Table 1). Of these 56 elements, 51 corresponded to known genes, while the remaining 5 corresponded to expressed sequence tags. The major categories of genes that showed a significant increase in response to IFN-α treatment included genes encoding molecules involved in protein degradation, glucocorticoid attenuated response genes, GTP-binding/GTPase activity, cytokines and chemokines, transcriptional regulation, antigen presentation (MHC molecules), antiviral, RNA editing, and other regulatory proteins. However, several gene products can function in more than one category, such as myxovirus resistance 2 (Mx2) and several

TABLE 1. Genes stimulated by IFN-α in primary cultured neurons

Classification	Control (mean fluorescence intensity)	IFN-α (mean fluorescence intensity)	IFN-α/control (n-fold change)	Genbank accession no.	Description[a]
Ubiquitination/protease	−23.0	3,168.0	316.8	X56602	ISG15
	−8.7	2,776.0	277.6	AW047653	USP18
	32.8	634.4	19.3	U22033	Lmp7
	240.1	784.3	3.3	Y10875	Lmp10
Glucocorticoid attenuated response genes	−15.1	2,714.3	271.4	U43086	GARG-49
	6.7	1,953.2	195.3	U43084	GARG-16
	18.2	616.1	33.8	AV152244	G1p2; IFN-α-inducible protein
	−5.7	253.5	25.3	U43085	GARG-39
Guanylate-binding protein	0.8	1,935.8	193.6	AJ007971	IIGP protein
	9.2	934.1	93.4	AA914345	IFN-inducible GTPase
	−7.4	921.6	92.2	M55544	GBP-1
	17.5	1,249.0	71.5	AJ007970	GBP-2
	32.1	1,096.8	34.1	U53219	IGTP
	66.5	1,949.6	29.3	AW047476	GBP-3
	1.8	200.4	20.0	L38444	TGTP
	13.9	249.3	17.9	M21038	Mx1
	34.3	520.9	15.2	U19119	related to GBP; G-protein-like LRG-47
	53.5	663.9	12.4	AJ007972	GTPI protein
	97.9	457.2	4.7	J03368	Mx2
Chemokine/adhesion	4.0	926.9	92.7	M33266	Cxcl10 (formerly IP10)
	120.8	340.6	2.8	U12884	VCAM
	414.4	947.9	2.3	M84487	VCAM
Regulatory	−17.9	726.5	72.6	AA959954	Ifih1
	2.2	650.8	65.1	L32973	Tyki
	−17.2	232.4	23.2	X67809	Ppicap
	21.6	109.6	5.1	AA760613	Lgals8
Transcription factor	39.6	1,342.8	33.9	U06924	STAT1
	−47.4	219.0	21.9	AW121732	IFN-induced protein 35 homolog
	24.9	535.4	21.5	L27990	Trim21
	36.7	510.2	13.9	D63902	Trim25
	51.8	607.4	11.7	U51992	ISGF3γ (IRF-9)
	−19.3	87.9	8.8	J03776	Trim30
	29.8	217.5	7.3	U73037	IRF7
	103.3	502.1	4.9	AA138192	Trim21
MHC and related genes	30.2	957.2	31.7	M35244	H2-T10; H2 T region locus 10
	2.6	204.3	19.2	Y00629	H2-T23; H2 T regions locus 23
	74.1	1,157.0	15.6	M35247	H2-T17; H2 T region locus 17
	99.3	1,332.0	13.4	U60020	Tap1
	26.1	215.0	8.2	U60091	Tap2
	433.3	2,188.4	5.1	X01838	β2m
	125.5	410.6	3.3	M18837	H2-Q1; H2 Q region locus 1
	140.4	440.9	3.1	X58609	MHC Class I antigen
	157.5	469.8	3.0	U33626	Pml
	120.3	352.9	2.9	M27034	H2-K; H2 K region
Antiviral	2.1	159.3	15.9	M31419	Ifi204
	29.2	203.7	7.0	U09928	PKR
	32.1	194.1	6.1	M31418	Ifi202b
	282.6	1,351.9	4.8	AW125390	Ifitm31
RNA editing	109.8	430.7	3.9	AW124271	Pnpt1
	25.5	82.7	3.2	U22262	Apobec 1
	117.1	376.9	3.2	AF052506	Adar
Unknown/EST	13.4	1,191.5	88.7	AA204579	EST
	79.6	1,873.7	23.5	AA816121	EST
	121.5	1,144.6	9.4	AI508931	EST
	183.5	517.9	2.8	AW121646	EST
	47.0	130.0	2.8	C88243	EST

[a] Abbreviations: Adar, adenosine deaminase, RNA-specific; Apobec 1, apolipoprotein B editing complex 1; β2m, beta-2 microglobulin; GARG, glucocorticoid attenuated response gene; GBP-1; GTP-binding protein-1; Ifih1, interferon induced with helicase C domain 1; Ifi202b, IFN-activated gene 202B; Ifi204, IFN-activated gene 204; Ifitm31, IFN-induced transmembrane protein 3-like; IGTP, IGN-γ-induced GTPase; IRF7, interferon regulatory factor 7; ISG15, IFN-induced 15 kDa protein; ISGF3γ, IFN-dependent positive acting transcription factor 3γ; Lgals8, lectin, galactose binding, soluble 8; Lmp7, large multifunctional protease 7; Lmp10, proteasome subunit beta 8 (Psmb8); Mx1, Myxovirus resistance 1; Mx2, Myxovirus resistance 2; PKR, IFN-inducible dsRNA dependent protein kinase; Pml, promyelocytic leukenmia; Pnpt1, polyribonucleotide nucleotidyltransferase 1; Ppicap, peptidylprolyl isomerase C-associated protein; STAT1, signal transducer & activator of transcription 1; Tap1, transporter 1 (ATP-binding cassette) sub-family B; Tap2, transporter 2 (ATP-binding cassette) sub-family B; TGTP, T-cell specific GTPase; Trim21, tripartite motif protein 21 (Ro protein); Trim25, tripartite motif protein 25; Trim30, tripartite motif protein 30; Tyki, thymidylate kinase family member; USP18, ubiquitin specific protease 18; VCAM, vascular cell adhesion molecule 1; EST, expressed sequence tag.
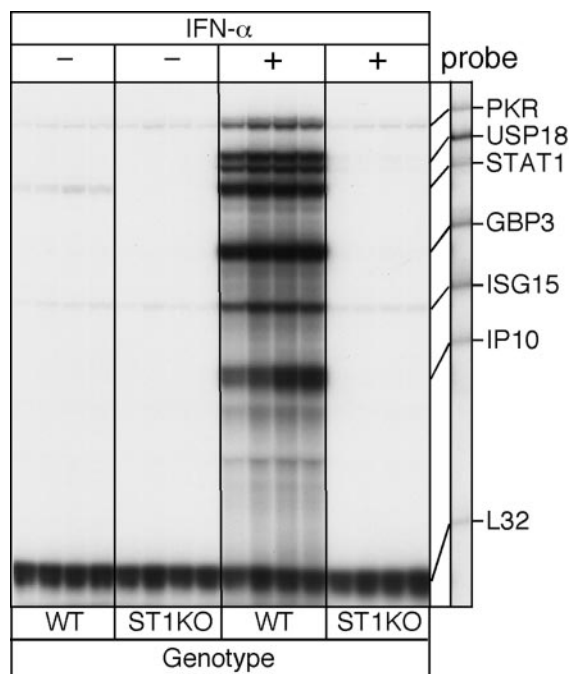
FIG. 1. The expression of a selected subset of IFN-stimulated genes in neurons. Multiprobe RPA analysis was performed on total RNA (5 μg/sample) extracted from primary cultured neurons prepared from the E15 embryonic brain of wild-type (WT) or STAT1 knockout (ST1KO) mice as described in Materials and Methods. Cultures were untreated or treated with murine IFN-α (250 IU/ml) or vehicle for 4 h prior to RNA extraction.

guanylate-binding proteins that have both antiviral and GTP-binding properties (41). Genes that showed the greatest increase following IFN-α treatment included IFN-induced 15-kDa protein (ISG15; 317-fold), USP 18 (278-fold), GARG-49 (271-fold), GARG-16 (195-fold), IFN-inducible GTPase (194-fold), and IP10 (CXCL10) (93-fold). Consistent with our previous finding in transgenic mice with astrocyte-targeted production of IFN-α (43), the expression of two key transcription factor genes participating in IFN-α/β signaling, STAT1 and ISGF3γ (p48 or IFN regulatory factor 9), was also enhanced by more than 10-fold after stimulation by IFN-α.

In contrast to these positive effects of IFN-α, our analysis failed to identify any genes whose expression was significantly decreased in IFN-α-treated neurons in comparison to untreated controls. Finally, to investigate the dependence of IFN-α-stimulated gene expression on STAT1, we examined neuronal cultures derived from mice with a targeted disruption of the STAT1 gene (25). The result of this analysis revealed a complete failure of IFN-α to alter significantly the expression of any genes in these cells. This finding indicated there is an obligatory role for STAT1 in the neuronal response to IFN-α.

To further confirm the results obtained with microarray analysis, we next performed RPA with probes constructed for a selected subset of the gene transcripts found to be increased by microarray analysis, including ISG15, USP18, STAT1, GBP3, protein kinase R (PKR), and CXCL10. In neurons from wild-type mice, low constitutive expression of the PKR, STAT1, and ISG15 genes was detectable (Fig. 1); however,

expression of these as well as all the other genes examined was highly upregulated following exposure to IFN-α. In neurons from STAT1 knockout animals, while there was low constitutive expression of the PKR and ISG15 genes, there was no effect of IFN-α on the level of expression of these or the other genes. Thus, these RPA findings further highlighted the marked STAT1-dependent response of neurons to IFN-α and, importantly, validated the findings obtained by microarray analysis.

Expression of interferon-regulated genes in GFAP-IFN-α and LCMV-infected mice. To extend these in vitro findings and further confirm the neuronal response to IFN-α and viral infection in vivo, we next examined the expression of the selected upregulated IFN-α-stimulated genes described above in the brain from mice either with astrocyte-targeted expression of an IFN-α transgene (1) or infected intracranially with the virus LCMV (8).

GFAP-IFN-α transgenic mice represent a relevant in vivo counterpart to the IFN-α-treated neuronal culture model. A multiprobe RPA survey of the IFN-regulated genes PKR, USP18, STAT1, GBP3, ISG15, and CXCL10 revealed a significant transgene dose-dependent increase in the expression of all these genes compared with wild type (Fig. 2A). Thus, in the GIFN39 brain, with higher levels of IFN-α expression, the transcriptional responses were considerably higher than in brain from the lower-IFN-α-expressing line, GIFN12. By in situ hybridization, in GFAP-IFN-α mice, RNA transcripts for the ISG15 gene were upregulated in various regions throughout the brain (Fig. 2B). Similar to the RPA results, levels of ISG15 RNA were higher in brains from the GIFN39 line compared with the GIFN12 line, with ISG15 highly expressed in the granular neuron layer of the cerebellum, hippocampus, and olfactory bulb (Fig. 2B, arrows). In the hippocampus, microscopic examination revealed that ISG15 transcripts were localized in the pyramidal (Fig. 2D, white arrow) and dentate gyrus granule (Fig. 2D, white arrowhead) neuron layers. While in the cerebellum, ISG15 transcripts were localized predominantly to the granule neuron layer of the cerebellum (Fig. 2E). Together, these data indicated that neurons are highly responsive to IFN-α in vivo.

Mice infected intracranially with LCMV have previously been shown to have increased expression of both IFNs-α/β and IFN-γ genes in the brain (36). Analysis of the brain by RPA revealed a similar response to that found in neuronal culture in vitro (Fig. 3A). While PKR, STAT1, GBP3, and ISG15 RNA transcripts were detectable at low levels in normal brain, the level of all these transcripts increased markedly by day 3 after LCMV infection (Fig. 3A). Much higher expression of these genes as well as CXCL10 was found at day 6 compared with day 3 following intracranial LCMV infection, which is consistent with the expression profile of the IFNs-α/β found in the brain of mice infected with LCMV (36).

To further determine the regional and cellular distribution of the IFN-regulated genes in LCMV-infected brain, we again examined ISG15. In situ hybridization revealed that ISG15 was upregulated in several brain regions, in particular, at 6 days after LCMV infection (Fig. 3B). In contrast with brain from GFAP-IFN-α mice, in LCMV-infected brain the pattern of expression of ISG15 was much more restricted, being localized predominantly in the meninges, choroid plexus, ependyma,
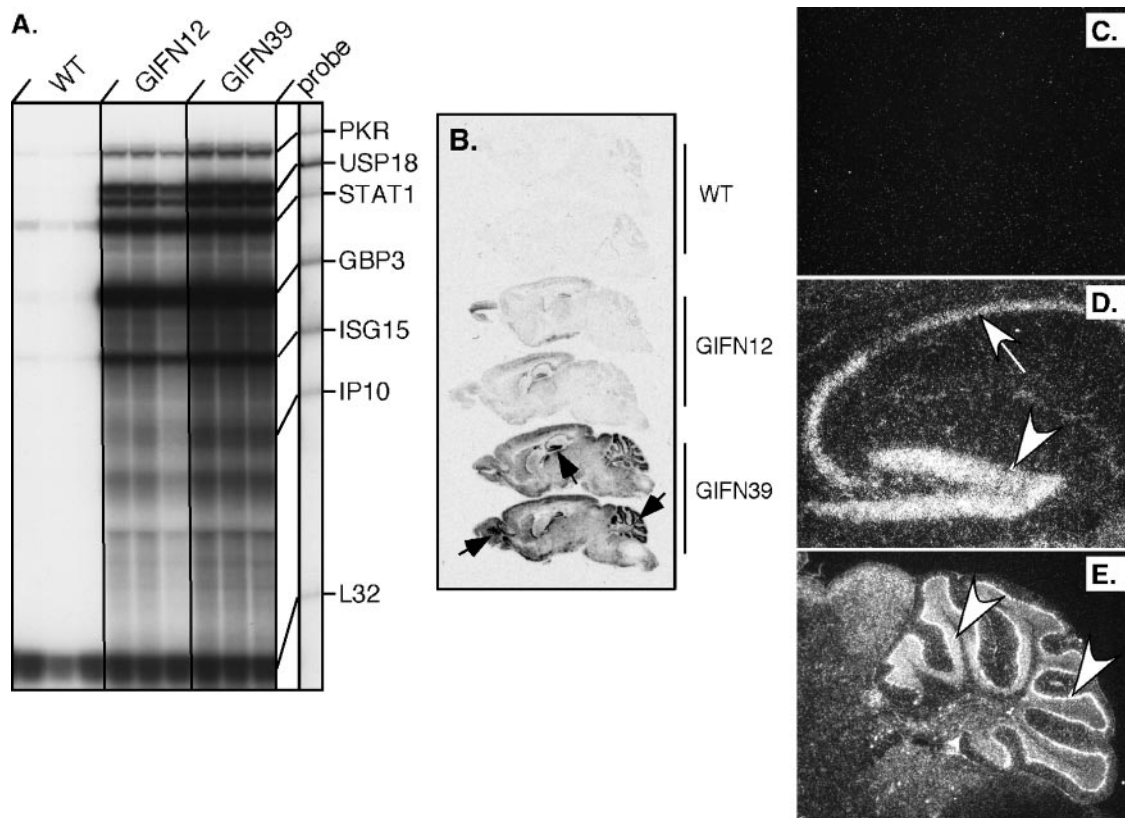
FIG. 2. The expression of IFN-stimulated genes in the brain of GFAP-IFN-α mice. (A) Multiprobe RPA analysis performed on poly(A)$^+$ RNA (1 μg per sample) extracted from the brain of 12-week-old mice. (B) In situ hybridization for ISG15 RNA in sagittal sections of the brain from wild-type, GIFN12, and GIFN39 (arrows) mice. (C to E) In situ hybridization localization of ISG15 RNA in the brain from wild-type (C) and GIFN39 (D and E) mice. Magnification, ×100 (C and D) and ×40 (E).

hippocampus, and olfactory bulb. This pattern of expression overlaps with the known principal sites of LCMV infection in the brain (1, 3). Although the distribution of ISG15 RNA was more restricted in the LCMV-infected brain, microscopic examination revealed that the expression of this gene did occur in some selected neuronal populations such as the granular neurons in the dentate gyrus (Fig. 3D, arrow).

DISCUSSION

In this study, we used the Affymetrix murine U74Av2 gene chip to analyze the global transcriptional profile of primary cultured neurons following exposure to IFN-α. A total of 56 genes (51 known genes and 5 expressed sequence tags) were increased significantly in expression by twofold or more. Genes that showed the greatest increase after IFN-α stimulation belonged to many different classes and included those encoding products involved in protein ubiquitination, glucocorticoid attenuated responses, guanylate-binding activity, transcription regulation, antigen presentation, and antiviral action. The overall response of neurons to IFN-α showed absolute dependence on STAT1 signaling since no significant change in IFN-α-stimulated gene expression occurred in the absence of this transcription factor. These gene chip results were validated by RPA for six highly upregulated genes and also confirmed the central importance of STAT1 in the transcriptional activation

by IFN-α. In addition, similar to IFN-α treated neurons, the expression of several IFN-α/β-stimulated genes was also increased in the brain and specific neurons of mice with either astrocyte-targeted expression of an IFN-α transgene or with LCMV infection. In fact, the findings from the GFAP-IFN-α mice here and in our previous studies (7) suggest that various neuronal populations, compared with other neural cells such as astrocytes, are hyperresponsive to IFN-α. Thus, in sum, our in vivo findings substantiated the in vitro culture observations and indicated that CNS neurons are highly responsive to IFN-α.

A comparison of our findings here for IFN-α treated neurons with those from recent studies employing similar global gene microarray approaches in the brain of mice with Sindbis virus encephalitis (19, 22) or monkeys with simian immunodeficiency virus-induced encephalitis (33) reveal there is considerable overlap with genes belonging to the ubiquitination pathway, MHC, transcriptional regulatory factors, glucocorticoid attenuated response genes, antiviral genes, and GDP-binding proteins which were all upregulated significantly. In fact, a comparison of our findings in the present study with those reported for mice infected with Sindbis virus (22) revealed that more than 90% of the genes significantly upregulated in neuronal cultures by IFN-α were also upregulated in the Sindbis virus-infected brain. These observations suggest that these genes may form the core of a common neuronal defensive response against viral infection in the brain. In support of this,
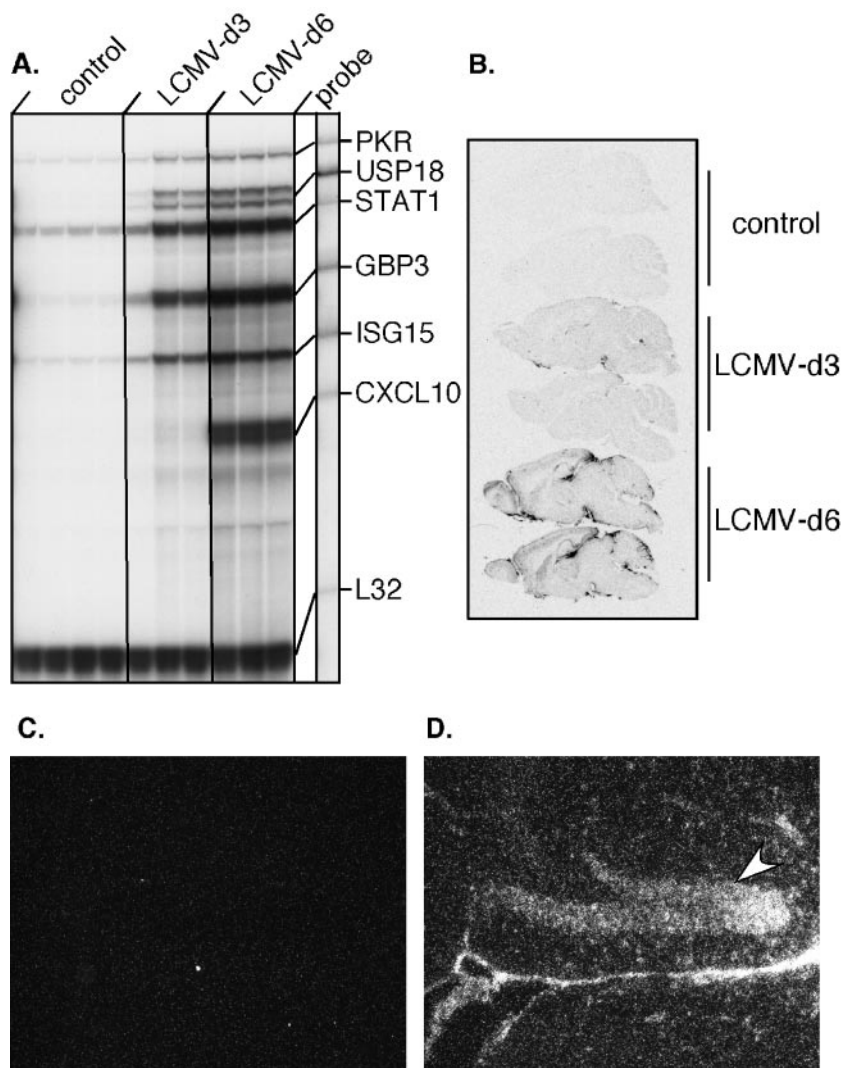
FIG. 3. The expression of IFN-stimulated genes in LCMV-infected brain. (A) Multiprobe RPA analysis performed on poly(A)$^+$ RNA (1 μg per sample) extracted from the brain of mice after intracranial inoculation of 250 PFU of LCMV. (B to D) In situ hybridization localization of ISG15 RNA in the brain from control (B and C) or LCMV-infected mice (B and D). Magnification, ×100 (C and D).

a number of these genes were shown to be expressed by neurons during viral infection, for example, ISG-15 (shown by the present study) and IP-10 (4) or STAT1 (7, 33) reported by previous studies. It is therefore also likely that the IFNs-α/β were responsible for the activation of these genes during these viral infections of the brain. In the case of LCMV, it has been shown previously that IFN-β and IFN-α are induced in the brain (36).

Further comparison of our findings with previously published studies profiling the IFN-α-regulated transcriptome of nonneural cell types indicates that, while there is some overlap, notable differences also exist in the cellular responses to this cytokine. Thus, almost 50% of the genes significantly upregulated in neuronal cultures by IFN-α were also reported to be upregulated in human fibrosarcoma cells. The total number of genes whose expression was upregulated in response to IFN-α was nearly threefold higher in human fibrosarcoma cells (12) than in murine neurons. In addition, while Der and coworkers

(12) reported that the expression of a number of genes was also downregulated in IFN-treated human fibrosarcoma cells, we did not observe such a response in murine neurons. Notwithstanding the many variables between these studies, including cell differentiation state (transformed versus primary) and species differences, duration of IFN-α treatment, and different gene chips used, this comparative analysis serves to reinforce the previously made point (37) that responses to IFN-α are cell-type specific.

The known functions of the majority of the gene classes found to be significantly increased in IFN-α-treated neurons include a prominent role in antiviral defense. Thus, a number of these genes encode proteins that can directly inhibit viral replication. For example, the RNA-dependent PKR is activated by viral double-stranded RNA (dsRNA) and phosphorylates the translational initiation factor eIF2, which then halts cellular translation, thus inhibiting host and viral protein synthesis in infected cells (34, 46). Similar to PKR, the RNA

editing enzyme adenosine deaminase, ADAR1, is activated by viral dsRNA and catalyzes hydrolytic C-6 deamination of adenosine to yield inosine, destabilizing the dsRNA helix by disrupting base pairing (35). The guanosine triphosphatases (GTPases) including Mx1 and Mx2 inhibit the replication of certain RNA viruses by binding to viral ribonucleoprotein structures and preventing transcription of viral RNA or translocation of viral subparticles within the cell (31). The ubiquitin ISG-15 is a plurifunctional molecule that, in addition to conjugation to proteins (20), can have immunostimulatory and direct antiviral actions. In complementing the direct innate antiviral response by neurons, many of the other classes of genes whose expression was significantly upregulated in neurons by IFN-α have functions that are linked to the antiviral immune response. For example, the genes encoding components of the ubiquitination and proteosomal pathways together with the MHC genes have a central role in viral protein degradation, peptide processing, and antigen presentation (47). The chemokine gene CXCL10 is linked to the chemoattraction of effector antiviral T-lymphocytes from the periphery to the CNS during viral infection (13). In all, our findings indicated that the majority of genes stimulated in neurons by IFN-α were clearly associated with the antiviral response.

We identified members of two gene families whose expression was strongly stimulated in neurons by IFN-α but whose functions are largely unknown. The first family, the glucocorticoid attenuated response (GARG) genes, GARG16, GARG39, and GARG49, encode proteins that are highly conserved across species and contain multiple tetratricopeptide repeat domains (39). Tetratricopeptide repeat domains are involved in specific protein-protein interactions in a variety of cellular processes that include ubiquitination, transcriptional regulation, and translocation of proteins within the cell (5). The second gene family consisted of the tripartite motif protein (TRIM) genes, TRIM21, TRIM25, and TRIM30. The products of this gene family also contain highly conserved structural domains consisting of the RING/B-box/coiled-coil triple motif structure involved in protein-protein interactions. There is evidence to suggest that the RING/B-box/coiled-coil triple motif containing molecules can form intracellular molecular scaffolds, is involved in transcriptional repression, and possesses E3 ubiquitin protein ligase activity (6). Given their prominence in the IFN-response and highly conserved and specialized structure, it will be of considerable interest in future studies to determine the roles of the respective GARG and TRIM family member genes in neurons and other cells.

Our finding that the neuronal transcriptional response to IFN-α was completely dependent on STAT1 was unexpected since STAT1-independent cellular responses to IFNs-α/β have been reported for other cell types. Such STAT1-independent responses include the inhibition of interleukin-7-stimulated proliferation of B cell lineage progenitors through induction of the Fas death domain-associated protein, Daxx (17), and the induction of IFN-γ gene expression by splenic mononuclear cells (28). Moreover, we showed that the absence of STAT1 markedly exacerbated IFN-α-mediated neurodegeneration within the CNS (43). The STAT1-independent nature of these IFN responses can be ascribed to the activation of alternative IFNAR-coupled signal transduction pathways. For example, the ability of IFN-α to induce IFN-γ gene expression is due to the activation of STAT4 in leukocyte mononuclear cells (28, 42). The absence of IFN-α-mediated transcriptional responses by neurons lacking STAT1 suggests that such alternative signal transduction pathways are not coupled to the IFNAR in these cells. A further implication from these findings is that the exacerbation of IFN-α-mediated neurodegeneration found in the CNS in the absence of STAT1 is unlikely to be due to the direct toxic actions of IFN-α on neurons but, rather, might involve the STAT1-independent response of other neural cells such as the glia. In support of this notion, in recent studies we have established that in contrast with neurons, glial cells exhibit a wide-ranging IFN-α-stimulated, STAT1-independent transcriptional response (S. Ousman, J. Wang, and I. L. Campbell, unpublished data).

The local production of IFNs-α/β in the CNS may provide an effective means for limiting viral infection and thereby protecting the host against potentially catastrophic consequences that would result from the injury or loss of neurons. From the genome-wide transcriptional profile generated by our findings, it is evident that neurons are a primary target for the actions of the IFNs-α/β and that these cells respond with a robust STAT1-dependent upregulation in the expression of a wide range of antiviral and host response genes. Therefore, we conclude that under the command of the IFNs-α/β, neurons themselves posses the innate potential to defend against viral infection in the brain.

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