Differential Effects of Rabies and Borna Disease Viruses on Immediate-Early- and Late-Response Gene Expression in Brain Tissues

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In situ hybridization and Northern blot analysis were used to examine expression of the immediate-early-response genes (IEGs) *egr-1*, *junB*, and *c-fos*, and the late response gene encoding enkephalin in the brains of rats infected intranasally with Borna disease virus (BDV) or rabies virus. In both Borna disease and rabies virus infections, a dramatic and specific induction of IEGs was detected in particular regions of the hippocampus and the cortex. Increased IEG mRNA expression overlapped with the characteristic expression patterns of BDV RNA and rabies virus RNA, although relative expression levels of viral RNA and IEG mRNA differed, particularly in the hippocampal formation. Furthermore, the temporal relationship between viral RNA synthesis and activation of IEG mRNA expression in BDV infection differed markedly from that in rabies virus infection, suggesting that IEG expression is upregulated by different mechanisms. Expression of proenkephalin (pENK) mRNA was also significantly increased in BDV infection, whereas in rabies virus infection, pENK mRNA levels and also the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were reduced at terminal stages of the disease, probably reflecting a generalized suppression of cellular protein synthesis due to massive production of rabies virus mRNA. The correlation between activated IEG mRNA expression and the strong increase in viral RNA raises the possibility that IEG products induce some phenotypic changes in neurons that render them more susceptible to viral replication.

The effector mechanisms involved in neurological damage induced by infection with neurotropic viruses are not fully understood. In many cases of virus-induced encephalopathy, the virus does not directly destroy neurons but causes indirect damage by triggering cell-mediated immune responses within the central nervous system (CNS) (3, 5, 12, 28, 31, 37, 41). Neurotoxins (30), free radicals (20), and proinflammatory cytokines (34) produced by infiltrating immune cells are thought to play an important role in the process of neuronal destruction. However, in addition to the involvement of components of the immune system in virus-induced neuropathology, viruses may trigger pathogenic mechanisms directly by altering neuronal functions. Impairment of neuronal functions has been reported for several neurotropic viral infections (2, 14-16, 19, 21-23, 25, 42). Abnormal levels of mRNAs of neurotransmitters have been found in brains infected with lymphocytic choriomeningitis virus (22) and Borna disease virus (BDV) (23). Rats and mice infected with Venezuelan equine encephalomyelitis virus showed a significant decrease in choline acetyltransferase activity in their brains (2, 21). In addition to altering neurotransmitter expression, viruses can also have selective effects on the electrophysiological activity of neurons. For example, rabies virus infection, which is characterized by only minor histopathological changes in the CNS, induces significant electrophysiological and sleep alterations (15, 16).

A large body of evidence has recently accumulated to demonstrate that neurotransmitters, growth factors, and mem-

brane electrical activity can modulate the expression of neuronal genes implicated in maintaining the adaptive plasticity of the nervous system. These genes have been identified as immediate-early-response genes (IEGs) which are transiently activated in the absence of intervening protein synthesis and, within minutes, encode proteins such as transcriptional factors that are commonly involved in the growth and development of dividing cells (7, 18, 36, 38). In the CNS, IEGs are believed to be important regulators of nerve cell responses and might be involved in long-term plastic changes in the brain (e.g., long-term potentiation). Regulatory proteins encoded by IEGs can also control the expression of late-response genes which encode more specific gene products, such as neuromodulatory peptides. For example, Fos and Jun gene products activate transcription from the enkephalin promoter (7, 36).

IEGs such as *fos* are activated during experimental ischemia, epilepsy, seizures, and injury, indicating that the physiological response to trauma has a genomic component. In the present study, we examined the possibility that neuronal damage and alteration in CNS function during neurotropic viral infections might also be associated with changes in the expression of IEGs and late-response genes. BDV and rabies viruses were chosen because both are highly neurotropic but differ considerably in their pathogenesis: rabies virus causes an acute lethal encephalomyelitis with only minor inflammatory reaction (16), whereas infection with BDV results in persistent CNS infection characterized by massive infiltration of inflammatory cells (5, 28). In situ hybridization and Northern (RNA) blot techniques were used to monitor the expression of the IEGs *egr-1*, *c-fos*, and *junB* as well as a late-response gene that encodes proenkephalin (pENK) in rat brains after infection with either rabies

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FIG. 1. Distribution of BDV RNA, *egr-1* mRNA, pENK mRNA (ENK), and G3PDH mRNA in adjacent coronal sections through the caudate putamen (x) and hippocampus (xx) of normal rats (a) and BDV-infected rats at 14 (b), 17 (c), and 21 (d) days after infection. RNA was detected by in situ hybridization on slide-mounted tissue sections as described in Materials and Methods.

virus or BDV. Our results provide the first evidence that the expression of IEG mRNA is strongly increased in the brains of rabies virus and BDV-infected rats, although the kinetics of IEG expression differs during each virus infection. Furthermore, pENK mRNA expression is upregulated in BDV-infected brains but dramatically reduced in rabies virus-infected brains.

MATERIALS AND METHODS

Virus infection of rats. Six-week-old female Lewis rats (Harlan Sprague-Dawley) were anesthetized with methoxyflurane (Metofane; Pittman-Moore, Inc., Mundelein, Ill.) and infected intranasally with 30 μ l of a 20% rat brain suspension containing 3×10^6 focus-forming units of BDV or with 30 μ l of a 10% mouse brain suspension containing 2.4×10^5 50% mouse intracerebral lethal doses of rabies CVS-24 virus. At the indicated times after infection, rats were euthanized and their brains were processed for in situ hybridization or RNA extraction.

RNA extraction and Northern blot analysis. Brains were collected and immediately placed in 2 ml of 4 M guanidine isothiocyanate containing 50 mM Tris-HCl (pH 7.2), 10 mM EDTA, and 1% β -mercaptoethanol. RNA extraction and Northern blot hybridization were performed as described previously (1). Briefly, total RNA was denatured with 10 mM sodium phosphate buffer, pH 7.4–50% (vol/vol) formamide at 65°C for 15 min and electrophoresed in a 1.2% agarose gel containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer (pH 7.4). RNA was transferred and covalently fixed onto nylon membranes (Fisher Scientific, Pittsburgh, Pa.). Hybridization probes were prepared from rabies virus N cDNA (13), BDV pAB5 cDNA (26), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (39), or pENK cDNA (33) and labeled with 32 P, using the multiprimer DNA labeling system (Amersham, Arlington Heights, Ill.) (specific activity, 10^9 cpm/mg).

Preparation of cRNA probes. The following riboprobes were prepared. (i) The BDV riboprobe was derived from pAB5 cDNA (a generous gift from I. Lipkin, University of California, Irvine), which contains incomplete nucleotide sequences of the

BDV 40-kDa protein (26), by cloning into pGEM-3Z. The resulting plasmid pGEM-BDV was linearized with *Eco*RI and transcribed from the SP6 promoter. (ii) The rabies virus N riboprobe was derived from plasmid pN1R2 (13), which contains the rabies virus N protein gene, by digestion with *Pst*I and *Xba*I and cloning into pGEM-3Z. The resulting plasmid pGEM-RN was linearized with *Eco*RI and transcribed with SP6 polymerase. (iii) The rat *c-fos* riboprobe was generated from plasmid pBS-c-fos (8) by digestion with *Xho*I and transcription with T3 polymerase. (iv) The *junB*-specific riboprobe was generated by cloning a *Bam*HI-*Eco*RI fragment of a cDNA clone (32) into pGEM-3Z. The resulting plasmid pGEM-junB was digested with *Bam*HI and transcribed with T7 polymerase. (v) The *egr-1* riboprobe was generated from a Bluescript plasmid containing mouse *egr-1* cDNA (4) by digestion with *Hind*III and transcription with T7 polymerase. (vi) The pENK riboprobe was generated from plasmid pGEM-3-ENK (33) by digestion with *Hind*III and transcription with T7 polymerase.

In situ hybridization. Slides of frozen unfixed tissue sections were prepared as described by Herkenham and Pert (17). Tissue pretreatment and hybridization were carried out as described previously (40). 35 S-labeled riboprobes were used for the detection of rabies virus RNA, BDV RNA, *egr-1* mRNA, *junB* mRNA, *c-fos* mRNA, and pENK mRNA. The G3PDH-specific hybridization probe was prepared by nick translation of a 983-bp fragment of the G3PDH cDNA. After hybridization, slides were either exposed to Hyperfilm- β Max (Amersham) for 4 days or coated with Kodak NTB2 emulsion and exposed for 4 weeks in light-tight boxes at 4°C.

pENK mRNA synthesis was assayed quantitatively on digitized X-ray autoradiograms, using an image analysis system (MCID system M4; Imaging Research Inc., St. Catharines, Ontario, Canada).

RESULTS

Expression of viral and IEG RNAs in brain tissues of BDV- or rabies virus-infected rats. In situ hybridization on coronal brain sections through the caudate putamen and hippocampal regions, respectively, was used to monitor the expression of

FIG. 2. Distribution of rabies virus N RNA (R-N), *egr-1* mRNA, pENK mRNA (ENK), and G3PDH mRNA in adjacent coronal sections through the caudate putamen (x) and hippocampus (xx) of normal rats (a) and rabies virus-infected rats at 48 (b), 72 (c), 96 (d), 120 (e), and 144 (f) h p.i. Hybridizations were performed on slide-mounted tissue sections as described in Materials and Methods.

viral RNA and mRNAs of *erg-1*, pENK, and G3PDH (Fig. 1 and 2). The data presented in Fig. 1 and 2 represent results from in situ hybridization of brain sections from one rat per time interval. Analyses from two other animals yielded similar results (data not shown). BDV RNA was readily detected in the basolateral cortex and in the hippocampus at 14 days (Fig. 1b) and at 17 days (Fig. 1c) postinfection (p.i.). However, at 21 days p.i., when animals exhibited severe neurological signs such as seizures, convulsions, and tremor, BDV RNA levels in the cortex and hippocampus had markedly increased (Fig. 1d). Similarly, a strong hybridization signal for *erg-1* mRNA was detected at 21 days p.i., particularly in the piriform cortex and hippocampal formation (Fig. 1d), although no clear signal was detected in normal or in BDV-infected brains at 14 and 17 days p.i. (Fig. 1 a to c).

Dark-field micrographs revealed a codistribution of BDV RNA and *egr-1* mRNA throughout the hippocampus (Fig. 3A and B) but definite region-specific differences in expression levels. For example, BDV RNA expression was relatively high in cornu ammonis 3 (CA3) and CA4 and relatively low in the dentate gyrus, whereas *egr-1* RNA levels were highest in the dentate gyrus but very low in CA3 and CA4. In the parietal cortex, the distribution of *egr-1* mRNA was similar to that of BDV RNA but the number of neurons expressing *egr-1* mRNA was much lower than that expressing BDV RNA (data not shown).

In situ hybridization of rabies virus-infected brain sections with a rabies virus-specific probe revealed the presence of rabies virus RNA in the piriform cortex at 96 h p.i. (Fig. 2d). At 120 h p.i., rabies virus RNA levels were nearly maximal throughout the grey matter of the brain, including cortex, caudate putamen, hippocampus, thalamus, and hypothalamus. The appearance of rabies virus RNA was accompanied by a

large increase in the expression of *egr-1* mRNA in the piriform cortex at 96 h p.i. (Fig. 2d) and in the hippocampus at 120 h p.i. (Fig. 2e).

Rabies virus RNA and *egr-1* mRNA were codistributed in the hippocampus (Fig. 3C and D) and, as in BDV-infected brain tissue, there were region-specific quantitative differences in the expression of both RNA species. Whereas rabies virus RNA expression was relatively high in CA3 and CA4, *egr-1* RNA levels were highest in CA2. In contrast to BDV-infected tissues, neither rabies virus nor *egr-1* RNA was detected in the dentate gyrus. In the parietal cortex, the distribution of *egr-1* mRNA was similar to that of rabies virus RNA but, as in BDV-infected brains, the number of neurons expressing *egr-1* mRNA was much lower than that of neurons expressing viral RNA. Unlike BDV-infected brains, there was some preferential expression of both rabies virus RNA and *egr-1* mRNA in the deeper layers of the cortex (data not shown).

Clear hybridization signals for *junB* mRNA and *c-fos* mRNA, respectively (Fig. 4c and d), in addition to the very strong signal for *egr-1* mRNA (Fig. 4b), were detected in the hippocampus and cortical areas of BDV-infected (21 days p.i.) and rabies virus-infected brains (120 h p.i.) but not in normal brain tissue. The relative expression levels and distribution patterns of *junB* mRNA (not shown) were very similar to those of *egr-1* mRNA. The distribution of *c-fos* mRNA showed less pronounced region-specific quantitative differences within the hippocampal formation.

Changes in expression of pENK mRNA in brain tissues of BDV- or rabies virus-infected rats. Sections of BDV-infected brains showed a modest increase in pENK mRNA expression at 21 days p.i. in the caudate putamen (Fig. 1d) and in the hippocampus (not shown) when clinical signs were at a peak. As quantitatively determined by image analysis of X-ray film

FIG. 3. Dark-field photomicrographs of coronal sections through the hippocampus of a BDV-infected rat (A and B) and a rabies virus-infected rat (C and D) probed for viral and *egr-1* RNA. BDV RNA (A) and *egr-1* mRNA (B) are codistributed throughout the hippocampus but show region-specific differences in relative expression levels, particularly in the dentate gyrus (DG) and in CA4 and CA3. In CA1 and CA2, relative expression levels are similar for both RNAs. In rabies virus-infected hippocampus tissue, rabies virus RNA (C) and *egr-1* RNA (D) exhibit an overlapping distribution but also regional differences in relative expression levels. Although rabies virus RNA levels are relative high in CA4 and CA3, *egr-1* RNA expression is highest in CA2. Note the absence of rabies virus RNA and *egr-1* mRNA in the DG. Exposure time, 4 weeks; scale bar, 200 μ m.

autoradiographs, the amount of pENK mRNA in the caudate putamen of BDV-infected rats was 86% higher than that in normal rats (Fig. 5). No notable differences between normal and BDV-infected brains were observed in sections hybridized with a G3PDH-specific probe which served as an internal control for noninducible gene expression.

In contrast to BDV-infected brains, the level of pENK mRNA in the caudate putamen of rabies virus-infected brains decreased markedly by 120 h p.i. (Fig. 2c, ENK) and at 144 h p.i., the expression of pENK mRNA was reduced to very low levels. Quantitative image analysis revealed an 85% reduction of pENK mRNA in the caudate putamen of rabies virus-infected brains at 144 h p.i. (Fig. 5). In addition to the reduction in pENK mRNA expression, G3PDH mRNA also decreased markedly starting at 120 h p.i.

The differential effect of infection with BDV or rabies virus on pENK and G3PDH mRNA expression was further demonstrated by Northern blot analysis (Fig. 6 and 7). Although pENK mRNA expression was increased in all five BDV-infected rats at 21 days p.i. (Fig. 6D), no obvious differences in G3PDH mRNA levels were detected between normal and BDV-infected brains (Fig. 6A and D). In contrast, Northern blot analysis of rabies virus-infected brains revealed a significant decrease in the amount of pENK mRNA at 96 h p.i. in all

five brains and only sparse amounts of this mRNA in three of five brains at 144 h p.i.; G3PDH mRNA levels were also significantly reduced at 144 h p.i. (Fig. 7D).

DISCUSSION

The present study demonstrates that IEGs encoding transcriptional factors are dramatically and specifically induced in certain neuronal populations of the brain after infection with rabies virus or BDV. Such a massive increase in the expression of primary-response genes in the brain has been previously observed only after experimental induction of generalized tonic-clonic seizures (7), after transient ischemia (7), or after *N*-methyl-D-aspartate (NMDA) receptor stimulation (29). In both rabies virus- and BDV-infected brain tissues, *egr-1*, *junB*, and *c-fos* expression colocalized with viral RNA expression particularly in the hippocampus and to a lesser extent in the cortical layer. Within the hippocampal formation, rabies virus RNA and *egr-1* mRNA were found in CA1, CA2, CA3, and CA4 but not in the dentate gyrus, whereas BDV RNA and *egr-1* mRNAs were detected in all formations of the hippocampus, including the dentate gyrus. Despite this colocalization of viral RNA and *egr-1* mRNA, relative expression levels differed, particularly in the dentate gyrus of BDV-infected brain tissue,

FIG. 4. Distribution of ^{35}S -labeled antisense probes for *egr-1* (b), *junB* (c), and *c-fos* (d) in adjacent sections through the hippocampus of BDV-infected rats at 21 days p.i., rabies virus-infected rats at 120 h p.i., and normal control rats. Row a shows hippocampal sections from a BDV-infected rat hybridized with a BDV RNA probe, from a rabies virus-infected rat hybridized with a rabies virus RNA probe, and from a normal rat hybridized with a BDV RNA probe, respectively. Hybridizations were performed on slide-mounted sections as described in Materials and Methods.

in which *egr-1* mRNA expression levels were highest but in which expression of BDV RNA was relatively low compared with that of other regions of the hippocampus. These differences between viral RNA and *egr-1* mRNA expression suggest that in addition to viral replication, other factors (e.g., cytokines, growth factors, and neurotrophins) produced during inflammatory processes contribute to the upregulation of IEG expression. This might be the case particularly in BDV infection, in which IEG activation is observed when inflammatory reactions are at a peak. Furthermore, the preferential induction of *egr-1* and *junB* in the dentate gyrus of BDV-infected brains, analogous to the induction of *c-fos* after acute NMDA administration (27), points to the possible involvement of excitatory amino acids in BDV-mediated IEG activation. Since NMDA induces seizures, it is possible that the enhanced IEG mRNA expression in BDV-infected brains is due to seizures commonly observed in the acute phase of BDV infection.

The temporal relationship of viral RNA and *egr-1* mRNA expression in BDV-infected brain tissue differs markedly from that in rabies virus-infected brain tissue. While activation of *egr-1* mRNA expression occurs concurrently with rabies virus RNA synthesis, *egr-1* mRNA expression in BDV-infected brain tissue is observed much later after BDV RNA synthesis, as detected by in situ hybridization. These differential effects on IEG mRNA expression may reflect differences in the mode of IEG activation during these viral infections. IEGs can be activated by electrical or chemical depolarization or by growth

FIG. 5. pENK mRNA levels in the caudate putamen of control, BDV-infected, and rabies virus-infected rats. Radioactivity (disintegrations per minute [dpm]) was determined by semiquantitative image analysis performed on digitized X-ray autoradiograms. Four sections were analyzed from each of the five control, four BDV-infected (21 days p.i.), and five rabies virus-infected (144 h p.i.) brains. The mean dpm value of control animals was taken as 100%. Statistical analysis (one-factor analysis of variance) revealed a significant increase in pENK mRNA levels in the caudate putamen of BDV-infected brain tissue (*, $P < 0.05$) and a significant decrease (*, $P < 0.05$) in pENK mRNA levels in the caudate putamen of rabies virus-infected brains. Bars over columns indicate standard deviations from mean values.

FIG. 6. Effect of BDV infection on expression of pENK and G3PDH mRNA. RNA was isolated from five normal rat brains (A) and five BDV-infected rat brains at 14 (B), 17 (C), and 21 (D) days p.i. Ten micrograms of brain RNA from each rat was analyzed by Northern blotting. BDV RNA, pENK mRNA (ENK), and G3PDH mRNA were identified by using specific 32 P-labeled hybridization probes as described in Materials and Methods.

factors (7, 18, 36, 38). Depolarization of neurons is a possibility during rabies virus infection, in which the expression of viral glycoprotein on the cell surface might lead to a disturbance of ion channels. The electrophysiological alterations observed in the brains of rabies virus-infected mice (15, 16) are consistent with such a possibility. In BDV infection, growth factors and neurotrophins produced at later stages of the disease as a consequence of inflammatory reactions (34) might account for IEG activation. In this context, it has been shown that activated macrophages produce free radicals (11) which can induce gene expression of nerve growth factor and basic fibroblast growth factor in astrocytes (29).

The physical and pathological role of transcription factors in CNS pathogenesis is not fully understood. Transcription factors such as *c-fos* are believed to serve as intermediates that regulate the expression of secondary-response genes by coupling neuronal signals to the transcription of target genes, e.g., the pENK gene, thus leading to an altered phenotype (7, 9, 18, 36, 38). Our observation that expression of pENK mRNA is upregulated in BDV-infected brains supports this hypothesis. Alternatively, the induction of transcription factors could be responsible for apoptosis of neuronal cells, since it has been shown that continuous *c-fos* expression precedes programmed cell death in vivo (35).

In rabies virus-infected brain tissues, *egr-1* mRNA and pENK mRNA levels were inversely related. Since expression of the noninducible housekeeping gene that encodes G3PDH is also markedly decreased in rabies virus-infected brains, the downregulation of pENK RNA expression likely reflects global suppression of cellular protein synthesis because of the extensive synthesis of rabies virus mRNA, which competes success-

FIG. 7. Effect of rabies virus infection on expression of pENK and G3PDH mRNA. RNA was isolated from five normal rat brains (A) and five rabies virus-infected rat brains at 72 (B), 96 (C), and 144 (D) h p.i. Ten micrograms of brain RNA from each rat was analyzed by Northern blotting. Rabies virus RNA, pENK mRNA (ENK), and G3PDH mRNA were identified by using specific 32 P-labeled probes as described in Materials and Methods.

fully for ribosome binding sites owing to its higher binding affinity (24). Such a generalized inhibition of protein synthesis would affect the transcription of a variety of cellular genes except for IEGs, in which transcription is independent of protein synthesis (7, 18, 36, 38).

One peculiar characteristic of rabies virus infection in vivo is the extremely low rate of viral replication during the first 72 h p.i., when viral RNA can only be detected after amplification by a reverse transcriptase-polymerase chain reaction (10). However, after 96 h p.i., rabies virus RNA levels increase exponentially in almost all areas of the brain, reaching maximum levels at 120 h p.i. This exponential increase in rabies virus RNA synthesis, as determined by in situ hybridization or Northern blot analysis, correlates with the increase in *egr-1* mRNA expression, raising the possibility that activation of IEGs promotes viral replication by inducing some phenotypic change in neurons that renders them more amenable to viral replication. Such a phenotypic alteration might also play a role in BDV infection, since BDV RNA levels are highest when *egr-1* mRNA expression is strongly increased. This hypothesis is supported by the recent finding that pretreatment of PC12 cells with neurotrophic factors, which induce biological and morphological alterations of these cells (7), resulted in a significant increase in the number of cells expressing viral antigens after BDV infection (6).

The possible relationship between IEG activation and progression of viral infection in the CNS could have therapeutic implications. For example, the use of antisense oligonucleotides to downregulate transcription of particular IEGs that are upregulated during viral infections can be envisioned.

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