Brain Research, 451 (1988) 333–339
Elsevier

BRE 13606

Viral infection of neurons can depress neurotransmitter mRNA levels without histologic injury

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(Accepted 8 December 1987)

Key words: Neurotransmitter; Somatostatin; Virus

Neonatal mice inoculated with lymphocytic choriomeningitis virus (LCMV) have non-lytic persistent neuronal infection and disturbed behavior. We now show that LCMV replicates in neurons containing the neurotransmitter somatostatin without morphologic evidence of injury and that persistent neuronal LCMV infection in mice is attended by a decrease in brain levels of somatostatin mRNA. Brain levels of mRNA for another neurotransmitter peptide, cholecystokinin, are not decreased. These data are the first to localize a virus to a specific neurotransmitter-containing cell during in vivo infection and suggest that persistent viral infections could cause neurologic or psychiatric diseases through selective effects on brain levels of neurotransmitter mRNAs.

INTRODUCTION

Several neurologic and psychiatric diseases are associated with characteristic neurotransmitter abnormalities[3,4,10,17,23,26,33,35,37,39]. The etiologies of most of these diseases are unknown and the mechanism by which neurotransmitter disturbances occur is unclear. One hypothesis holds that infectious agents, particularly viruses, may be involved in the pathogenesis of neurotransmitter disorders[1,18,27]. To address the role of viral infections in the pathogenesis of neurotransmitter diseases we are studying the effects of experimentally induced central nervous system (CNS) viral infections on neurotransmitter function. We have chosen to begin our analysis using a non-lytic virus, lymphocytic choriomeningitis virus (LCMV), which causes persistent neuronal infection and neurologic dysfunction without cytopathology in its natural host, the mouse[16,32]. In this paper we report two distinct morphologic patterns of virus-neurotransmitter interaction in brain: in one, individual somatostatin neurons also contain viral antigens; in the other, processes from uninfected somatostatin neurons communicate directly with LCMV-infected neurons. These phenomena are accompanied by a selective, significant decrease in whole brain levels of somatostatin mRNA.

MATERIALS AND METHODS

Mice, virus, antibodies and cDNA probes

BALB/WEHI mice were obtained from the breeding colony of The Research Institute of Scripps Clinic, La Jolla, CA. The Armstrong CA1371 strain of LCMV Clone 53b was propagated in BHK-21 tissue culture and quantitated by plaque assays in Vero cells[9]. Mice were infected through intracerebral (I.C.) inoculation within the first 18 h of life with 1000 plaque-forming units of LCMV. This establishes persistent infection for the lifetime of the animal without affecting its lifespan[6,15]. As described[6,28], mice were phlebotomized and assayed at random to insure that they were persistently infected. Antibodies to LCMV were prepared by inoculating guinea

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pigs with LCMV. The specificity of these antibodies was determined as reported[5]. Antibodies to somatostatin were prepared by immunizing rabbits with synthetic peptide CGGSANSNPAMAPRE coupled at the amino terminus to the carrier protein keyhole limpet hemocyamin (KLH). This sequence contains the neurotransmitter somatostatin 28(1–12), SANSNPAMAPRE, with an amino extension of two glycine residues as spacers and a terminal cysteine residue for coupling to KLH. The specificity of anti-peptide antibodies for native somatostatin was confirmed in experiments showing expected distribution of these antibodies in cell bodies and processes in murine brain and through blocking experiments with free somatostatin 28 (ref. 20). The cDNA probes for use in RNA analysis included rat pre-prosomatostatin, a gift from J. Habener (Harvard University, Boston, MA)[13], rat cholecystokinin obtained from R. Haun (Purdue University, West Lafayette, IN)[7] and 28S ribosomal RNA, contributed by P. Southern (Research Institute of Scripps Clinic, La Jolla, CA)[36]. The identity of these probes was confirmed by restriction mapping and hybridization in northern blots to RNA of appropriate size. In addition, the somatostatin probe was shown to hybridize to neurons in rat brain previously tagged immunohistochemically with antibody to somatostatin 28(1–12)[21]. Probes were labeled with ^{32}P via nick translation[31] to specific activity of $1–10 \times 10^8$ cpm/μg of DNA and were used in concentration of 1×10^8 cpm/ml of hybridization solution.

Immunohistochemistry

Mice were sacrificed at 4 months of age via cardiac perfusion with 4% paraformaldehyde. Brains were removed, cryoprotected for 18–24 h in 19% sucrose in phosphate-buffered saline (PBS), and sectioned coronally at 30-μm intervals in a cryomicrotome. Sections were incubated 12–16 h at 4 °C simultaneously with polyclonal guinea pig antibody to LCMV and polyclonal rabbit antibody to somatostatin 28(1–12). Primary antibodies were diluted 1:1000 in PBS, 0.3% Triton X-100 (J.T. Baker, Phillipsburg, NJ), 1 mg/ml bovine serum albumin. Sections were washed in PBS, incubated 1 h at 24 °C simultaneously with rhodamine (RITC)-conjugated goat anti-guinea pig Ig and fluorescein (FITC)-conjugated goat anti-rabbit Ig (Kirkegaard and Perry, Gaithersberg, MD). Secondary antibodies were diluted 1:500 in PBS, 0.3% Triton X-100. These antibody concentrations were selected on the basis of pilot experiments performed to determine conditions for optimal sensitivity and specificity. Sections were washed in PBS and wet-mounted on glass slides with PBS–glycerol under coverslips. Immunofluorescent photomicrographs were taken using filters to exclude either RITC or FITC fluorescence. Double exposure of film to include RITC and FITC fluorescence in single fields was used to detect coincidence of LCMV antigens and somatostatin. Uninfected mice, age- and sex-matched, were processed in parallel as controls. Brain sections were stained with hematoxylin and eosin (H & E) or Richardson's solution for light microscopic study.

RNA analysis

Brains were removed from 4-month-old virus-infected and uninfected mice and snap frozen in liquid nitrogen. Individual brains were homogenized in guanidinium thiocyanate using an Ultra Turrax (Tekmar Co., Cincinnati, OH), sonicated, then centrifuged over cesium chloride for 16–18 h at 23,000 rpm in an SW28 rotor (Beckman Instruments, Palo Alto, CA). RNA pellets were dissolved in sterile H_2O, extracted twice with phenol-chloroform, precipitated in NaCl and ethanol and then re-dissolved in sterile H_2O. RNA solutions were examined spectrophotometrically for RNA and protein concentration. All samples used showed 260/280 nm ratio greater than 1.9 insuring against protein contamination. For dot blot analysis, 12 μg aliquots of RNA were denatured at 65° for 15 min in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate), 7.4% formaldehyde, then serially diluted in 15 × SSC. Samples were applied to nitrocellulose membranes using a 96-well minifold dot blot apparatus (Schleicher and Schuell, Keene, NH). Nitrocellulose membranes were baked 2 h at 80 °C, prehybridized 4 h at 37 °C in 50% deionized formamide, 5 × SSC, 2.5 × Denhardt's solution, 100 μg/ml^{-1} boiled, sonicated salmon sperm DNA and then hybridized for 24 h at 37 °C with cDNA probe to rat pre-prosomatostatin. After hybridization, membranes were washed in 2 × SSC, 0.1% SDS at 37 °C for 30 min, in 0.1 × SSC, 0.1% SDS at 55 °C for 30 min, then exposed against Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at –70 °C with Cronex

lightning plus intensifying screens (Dupont, Wilmington, DE). Following exposure to film, membranes were washed in 0.1 × SSC, 0.1% SDS at 85 °C for 2 h to remove cDNA hybrids. Membranes were then reused in hybridization experiments with probes to rat cholecystokinin and 28S RNA. Conditions for prehybridization, hybridization, washing and autoradiography were as described above.

Films were scanned with an LKB Ultrascan laser densitometer (LKB, Sweden). Because multiple RNA samples were examined, we found it necessary to use more than one dot blot. In order to maintain an internal standard for these experiments and as an additional control to insure that mRNA levels were measured for equivalent amounts of total cellular RNA, neurotransmitter mRNA levels were measured in the context of individual 28S ribosomal RNA levels. For each RNA dot, we calculated a ratio of somatostatin hybridization intensity (SHI) and cholecystokinin hybridization intensity (CHI) to ribosome hybridization intensity (RHI) (SHI/RHI and CHI/RHI ratios, respectively). These ratios were then used to compare whole brain neurotransmitter mRNA concentrations in individual uninfected and LCMV infected animals. Pooled mouse spleen RNA was used as a negative control for the specificity of hybridization with probes to somatostatin and cholecystokinin.

In northern blot experiments, 2, 4 and 8 μg aliquots of brain RNA from uninfected and LCMV infected mice were denatured at 65 °C for 15 min in 50% deionized formamide, 6% formaldehyde, 0.5 × MOPS (1 × MOPS = 0.04 M morpholinopropane-sulfonic acid, 10 mM sodium acetate, 1 mM EDTA) and subjected to electrophoresis in 1% agarose, 6% formaldehyde gels. RNA was transferred to nitrocellulose membrane via capillary filtration with 20 × SSC. Thereafter, membranes were processed for hybridization and autoradiography as previously described for dot blots (above). Pooled mouse spleen RNA was again used as a control for specificity of hybridization with neurotransmitter probes.

RESULTS

The basis for using the LCMV-murine system was to learn the effects of a non-cytopathic persistent CNS infection on neurotransmitter function. Previous reports have shown no evidence of structural or ultrastructural CNS pathology in mice consistently infected with LCMV[32]. In our study as well, brain morphology was normal at the level of light microscopy. Multiple H & E- and Richardson's solution-stained sections through cortex, hippocampus, hypothalamus, cerebellum and brainstem were taken from 5 LCMV-infected mice for comparison to sections taken from identical anatomic regions in 5 uninfected mice. Sections from LCMV-infected brains were histologically similar to those from uninfected brains; specifically, LCMV-infected brain sections showed no evidence of neuronal dysmorphology, gliosis or inflammation.

Our first objective was to determine whether or not LCMV was tropic for somatostatinergic neurons. This was pursued in a double-label immunofluorescent system in which viral antigen was marked with FITC and somatostatin with RITC. Infected mice had LCMV antigens in neurons of the cerebral cortex, cerebellum, hippocampus, hypothalamus and striatum. As expected, uninfected animals failed to display LCMV antigens. The distribution of somatostatin immunoreactivity in brain was identical in uninfected and LCMV-infected animals. In accordance with earlier reports[24,25], somatostatin-containing cell bodies were found in dentate gyrus, hippocampus, hypothalamus and in layers II, III, V and VI of cerebral cortex. Somatostatin-immunoreactive processes were seen throughout the CNS. In LCMV-infected mice, analysis at the single cell level indicated that 10% of somatostatin-immunoreactive neurons also contained LCMV antigens. An example of the co-localization of LCMV antigens and somatostatin to a large striatal neuron with smooth dendrites is shown in Fig. 1A,B,C.

In addition to co-localization of viral antigens and somatostatin within single neurons, we noted multiple instances in which non-somatostatin-immunoreactive LCMV-infected neurons communicated with uninfected somatostatin-immunoreactive neurons. Fig. 1D shows a somatostatin-containing process from an uninfected cell in contact with a non-somatostatin-immunoreactive infected neuron.

Once we established that LCMV could infect somatostatin-immunoreactive neurons and cells in synaptic connection with somatostatin-immunoreactive neurons, we moved to consider the implications of

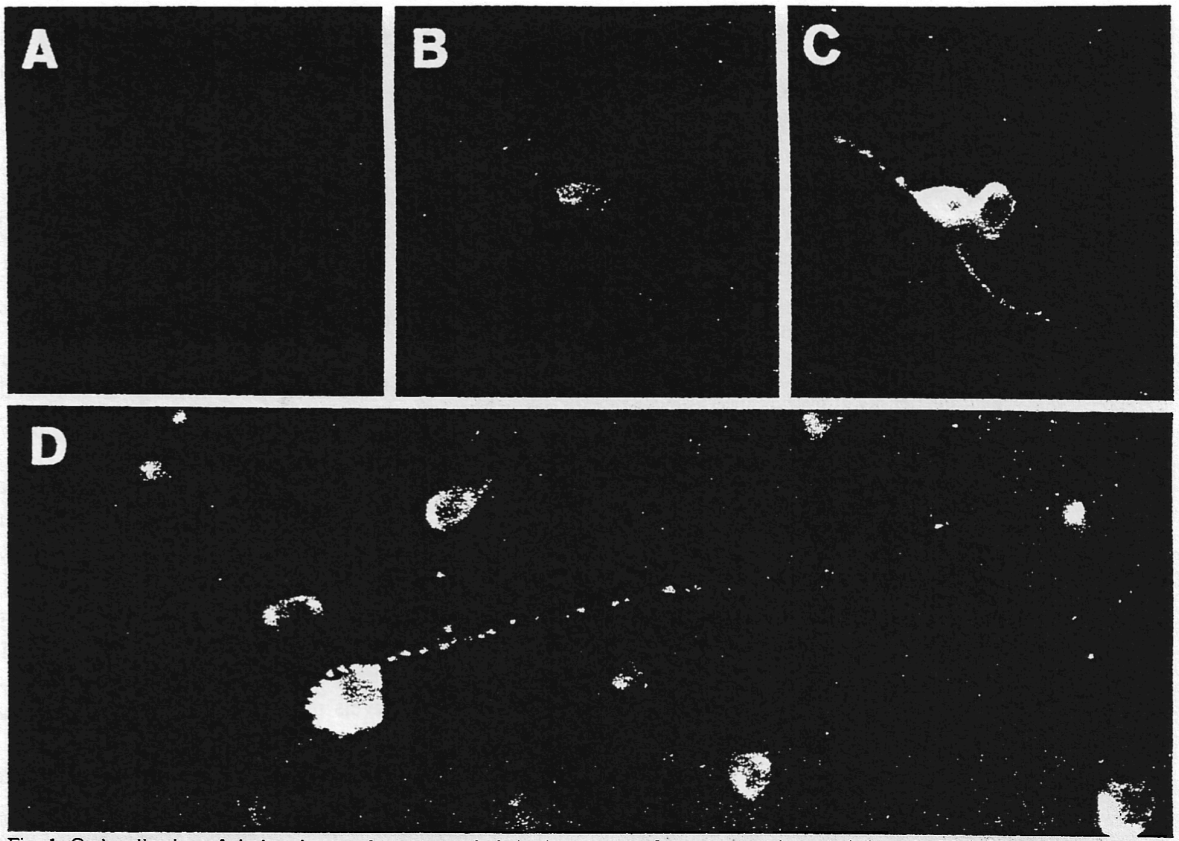

Fig. 1. Co-localization of viral antigen and somatostatin in brain neurons of a mouse persistently infected with LCMV antigen and somatostatin are shown in a striatal neuron with smooth dendrites (A.B.C). A: LCMV antigen (RITC). B: somatostatin (FITC). C: photographic superimposition of LCMV (RITC) and somatostatin (FITC) signal. Coincidence of RITC and FITC results in a yellow color with this technique. In D, a process from an uninfected somatostatin (FITC) is seen in contact with an LCMV-infected non-somatostatin neuron (RITC). The same method of photographic superimposition for C was employed for D.

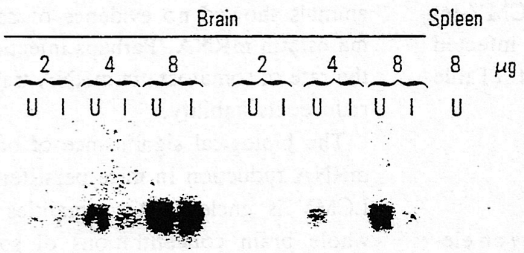

Fig. 2. Integrity of somatostatin mRNA extracted from brains of mice persistently infected with LCMV. Two Northern blot experiments are shown. Each lane received 2, 4 or 8 μg of total cellular RNA isolated from the brain of an uninfected (U) or an LCMV-infected (I) animal. The last lane, containing 8 μg RNA obtained from an uninfected mouse spleen, was included as a control for specificity of somatostatin probe hybridization.

persistent LCMV infection for neurotransmitter function. This was approached by measuring whole brain mRNA levels for somatostatin and another peptide neurotransmitter, cholecystokinin, in uninfected and LCMV-infected animals.

Hybridization analysis by dot blot and gel electrophoresis (northern blot) of RNA extracted from uninfected and LCMV-infected animals showed reduced brain levels of somatostatin mRNA in LCMV-infected animals as compared to somatostatin mRNA levels in matched uninfected controls (Table I). In contrast, when whole brain levels of cholecystokinin mRNA were similarly analyzed, no differences were observed between LCMV-infected and uninfected animals. Results of dot blot hybridization experiments with 10 uninfected and 10 LCMV infected animals are displayed in Table I. Mice persistently infected with LCMV had a greater than two-fold re-

TABLE I

Ratio of somatostatin mRNA and cholecystokinin mRNA to 28S ribosomal RNA in uninfected mice and mice persistently infected with LCMV

Mice were infected at birth with 1000 plaque-forming units of LCMV. Such mice remain persistently infected throughout their life. Ten uninfected and ten LCMV-infected age- and sex-matched 4-month-old mice were sacrificed, and their brains were removed, RNA-extracted, bound to nitrocellulose filters and hybridized with ^{32}P-labeled cDNA probes to somatostatin, cholecystokinin or 28S ribosomal RNA. Filters were exposed to film and film then analyzed via scanning laser densitometry to quantitate hybridization. To control for experimental variation, the density of hybridization signal with each neurotransmitter probe for each RNA extract was compared to the density of hybridization signal with the 28S ribosomal probe. This was used to calculate a ratio of neurotransmitter hybridization (SHI or CHI) to 28S RHI (see Materials and Methods). Data was analyzed by unpaired Student's *t*-test. S.E.M., standard error of the mean.

Experimental group	*Number*	*SHI/RHI*		*CHI/RHI*	
		Mean	*%S.E.M.*	*Mean*	*%S.E.M.*
Uninfected	10	2.17	±0.47	0.68	0.09
LCMV-infected	10	0.92	±0.14	0.95	0.18
		$P < 0.025$		$P < 0.2$	

duction in SHI/RHI ($P < 0.025$). Northern blot hybridizations confirmed this reduction in whole brain somatostatin mRNA with LCMV infection. Two representative northern blot experiments are seen in Fig. 2. Northern blots showed no evidence of somatostatin mRNA degradation or abnormal electrophoretic mobility in RNA extracted from LCMV-infected animals (Fig. 2). Mice persistently infected with LCMV showed no decrease in CHI/RHI (Table I).

DISCUSSION

Mice persistently infected with LCMV have an elevated threshold to noxious stimuli and demonstrate diminished locomotor activity with open field testing[16] yet manifest no structural CNS pathology[32]. We propose that this aberrant behavior reflects abnormal differentiated neuronal function. Our data suggest a selective lesion in whole brain levels of at least one neurotransmitter mRNA.

Immunohistochemically we have identified two ways in which persistent LCMV infection could affect CNS function. First, directly, through infection of neurons which elaborate a specific neurotransmitter (Fig. 1A,B); second, indirectly, through infection of neurons which communicate synaptically with uninfected cells producing that neurotransmitter (Fig. 1D). We have also shown that persistent LCMV infection causes a greater than two-fold decrease in whole brain levels of somatostatin mRNA (Table I, Fig. 2), without effecting a decrement in whole brain levels of mRNA for another peptide neurotransmitter, cholecystokinin.

The basis for somatostatin mRNA reduction in the CNS of persistently infected animals is uncertain. Given our finding that only 10% of somatostatin-immunoreactive neurons contained LCMV antigen, it is unlikely that most of the somatostatin mRNA reduction in these animals reflected infection of somatostatinergic neurons. The basis for somatostatin mRNA reduction may be infection of neurons which communicate synaptically with somatostatinergic neurons as illustrated in Fig. 1D. Alternatively, the effect of LCMV infection on somatostatin mRNA levels may be more complex, e.g. through modulation of other neurotransmitters which in turn directly or indirectly influence somatostatinergic neurons. We are currently exploring these possibilities by studying the effects of persistent LCMV infection on other neurotransmitter systems. Further, we do not know the molecular mechanism(s) by which somatostatin mRNA is reduced in infected animals. Gel electrophoresis of brain RNA from LCMV-infected animals showed no evidence of degradation of somatostatin mRNA. Perhaps infection has decreased the rate of somatostatin mRNA transcription and/or reduced its stability.

The biological significance of brain somatostatin mRNA reduction in mice persistently infected with LCMV is unclear. Abnormalities in regional and whole brain concentrations of somatostatin have been reported in Alzheimer's disease[33], Huntington's disease[26] and schizophrenia[26], human diseases characterized by profound impairment in intellectual and social function. Neurologic disturbances in our murine model of persistent CNS viral infection may reflect somatostatin dysregulation, alternatively, behavioral pathology may be mediated by abnormalities in different neurotransmitters, neurotransmitter receptors, or other components to CNS function.

Viral infections can cause disease through a variety of mechanisms including direct cytotoxicity, indi-

rect, host-mediated immune responses resulting in cytotoxicity and non-cytotoxic disturbance of differentiated cell function[29]. The last mechanism is particularly intriguing and may give insight into the pathogenesis of human neurologic and psychiatric disorders in which dysfunction is out of proportion to histopathology.

Evidence is emerging from several different infected animal model systems which document the potential for infectious agents to disturb differentiated cell function in the CNS. Hamsters inoculated i.c. with the scrapie agent have reduced tyrosine hydroxylase activity at the inoculation site prior to showing spongiform histopathology[8]. Mice infected i.c. with canine distemper virus become obese. Forebrain levels of dopamine and norepinephrine are reduced in these animals without histologic abnormality[22]. Recently, Barrett et al. found that mice inoculated i.c. with defective interfering variants of Semliki Forest virus have abnormal brain levels of neurotransmitter markers without dysmorphology[2]. Neurotransmitter mRNA levels have not been measured in these systems.

There is precedent in the mouse model of persistent LCMV infection for non-cytopathic interference with elaboration of differentiated cell products. In these earlier reports of endocrine dysregulation, as in the neurotransmitter disturbance we have just described, mRNA levels for the respective differentiated cell products were reduced.

In several mouse strains, persistent LCMV infection reduces circulating levels of thyroid hormone (TH)[19]. C3H mice inoculated at birth with LCMV have viral antigen in growth hormone (GH)-producing cells in the anterior pituitary, reduced pituitary levels of GH and manifest this deficiency clinically with runting and hypoglycemia[30]. TH mRNA and GH mRNA are reduced 2–5-fold in mice with TH deficiency[19] and GH deficiency[38], respectively. In none of these contexts is disturbed function accompanied by altered cell morphology or viability.

The implications of the data presented here for understanding human neurologic and psychiatric diseases is unclear. We and others have proposed that at least some of these diseases of unknown etiology, particularly those with associated neurotransmitter abnormalities are due to cryptic viral infections[1,18,27]. This hypothesis is based primarily on 3 lines of evidence. First, clinical and epidemiologic correlations between Parkinsonian syndromes and a variety of viral infections[1,18] and, more recently, serologic data linking bipolar depression to Borna Disease Virus infection[34]. Second, recognition of the category of slow CNS infectious diseases like Kuru and Creutzfeldt-Jakob, in which the inflammation characteristic of acute infection is conspicuously absent[11,12]. Third, demonstration of the persistence of viral genomic material in human brain by in situ hybridization[14]. We have now learned that viruses can persistently infect neurons and selectively alter levels of neurotransmitter mRNAs without cytopathology. The basis for neurotropism in such viral infections and the mechanisms by which neuronal luxury functions are disturbed will be critical subjects for future research.

ACKNOWLEDGEMENTS

This is Publication Number 4970-IMM from the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037. W.I.L. wishes to thank P.J. Southern and W. Reynolds for discussion of methodology and data. The authors thank Mrs. Diane Nolin for secretarial assistance. This work was supported in part by USPHS Grants NS-22347 (F.E.B. and E.L.F.B.), and NS-12428 and AG-04342 (M.B.A.O.). W.I.L. is a recipient of a CIDA from the NINCDS (NS-10126) and past recipient of a postdoctoral fellowship award from the National Multiple Sclerosis Society (MS FG-671-A-1).

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