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CHOLINERGIC SYSTEM IN EXPERIMENTAL RABIES IN MICE

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Summary. – A defect in cholinergic synaptic neurotransmission could explain the neuronal dysfunction that has been observed in rabies. The enzymatic activities of choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and enolase were assessed in the brains of rabies virus strain CVS-infected and uninfected mice. No statistically significant differences in activities of ChAT, AChE, or enolase were observed in the cerebral cortex or hippocampus of moribund CVS-infected mice versus controls. Binding to muscarinic acetylcholine receptors, which was assessed with ^{3}H-labelled quinuclidinyl benzylate (QNB), was also not significantly different in the cerebral cortex or hippocampus of CVS-infected mice and uninfected controls. The studies suggest that dysfunction of the cholinergic system is unlikely of fundamental importance in this mouse model of rabies.

Key words: acetylcholine; cholinergic system; encephalitis; pathogenesis; rabies; receptors

Introduction

Despite the dramatic and severe clinical neurologic signs and fatal outcome in human and animal rabies, the neuropathologic findings are usually quite mild (Perl and Good, 1991). In street rabies virus infection, there is a mild inflammatory response and eosinophilic cytoplasmic inclusions called Negri bodies may be present in infected neurons, but neuronal cytopathology is minimal. Since the morphologic changes do not explain the clinical illness in rabies, the infection must cause severe dysfunction of neurons.

Several RNA and DNA viruses have the ability to alter "luxury" or differentiated functions of cells without disturbing their vital or housekeeping functions (ability to survive) (Oldstone, 1988; 1989). This dysfunction may occur in the absence of pathologic changes. Viruses may impair production of endocrine hormones, lymphokines, and neurotransmitters. Studies performed *in vitro* have shown that rabies virus produces little or no inhibitory effect on cellular RNA and protein synthesis (Ermine and Flamand, 1977; Madore and England, 1977;

Tuffereau and Martinet-Edelist, 1985). Rabies virus infection of neurons could cause neuronal dysfunction by impairing synaptic neurotransmission.

A defect in cholinergic neurotransmission in the central nervous system in rabies has been hypothesized. Tsiang (1982) has reported a decrease in specific binding to muscarinic acetylcholine receptors in rabies virus-infected rat brains. In the present study the cholinergic system was examined in an experimental model in mice. The enzymatic activities of ChAT and AChE, which are required for the synthesis and degradation of acetylcholine, and enolase (a control enzyme that is not important for neurotransmission) were examined in challenge virus standard (CVS)-infected and uninfected mouse brains. In addition, specific binding to muscarinic acetylcholine receptors in CVS-infected and uninfected mouse brains was assessed with the ^{3}H-labelled antagonist, QNB.

Materials and Methods

Virus. The CVS-11 strain of fixed rabies virus was obtained from W. H. Wunner (Wistar Institute, Philadelphia, PA), and stock virus was grown in BHK-21 cells to a titer of 4.2×10^7 PFU/ml.

Animals. Six week-old female CD-1 mice (Charles River Canada) were used. Mice were inoculated in the left hindlimb footpad with 9.3×10^5 PFU of CVS in 0.03 ml PBS and 2 % foetal bovine serum.

Brain tissues. Eight to 11 days after inoculation with CVS, moribund CVS-infected and control mice were sacrificed by cervical dislocation. Brains were removed and dissected with the aid of a mouse brain slicer (Zivic-Miller Laboratories, Zelienople, PA) (see Jacobowitz, 1974). For enzyme assays, 5 % (w/v) homogenates of cerebral cortex and hippocampus were prepared in 400 mmol/l NaCl, 50 mmol/l sodium phosphate buffer pH 7.4, and 0.5 % Triton X-100. Aliquots were stored at -80 °C until they were assayed. Boiled tissue samples were used as controls for the enzyme assays. For binding assays, 10 % (w/v) homogenates of cerebral cortex and hippocampus were prepared in 0.32 mol/l sucrose in 10 mmol/l sodium phosphate buffer pH 7.4, and centrifuged at 14 000 x g for 15 mins. Pellets were stored at -80 °C until they were assayed.

Protein determinations were performed on all samples prior to enzyme and ^{3}H-QNB binding assays by the method of Peterson (1977) using bovine serum albumin in standards.

Assay for ChAT activity. ChAT activity was assayed in triplicate by the radiochemical method described by Fonnum (1975), and expressed as pmoles of acetylcholine formed/mg of protein/min (pmol/mg/min).

Assay for AChE activity. AChE activity was assayed in duplicate by the photometric method described by Ellman *et al.* (1961), and expressed as nmoles of acetylcholine degraded/mg of protein/min (nmol/mg/min).

Assay for enolase activity. Enolase activity was determined in duplicate using the method of Bergmeyer *et al.* (1983) as modified by Sigma Chemical Company (St. Louis, MO). The activity was assayed spectrophotometrically at 340 nm by coupling the reaction with pyruvate kinase and lactate dehydrogenase, which results in oxidation of NADH. The reaction was carried out at 25 °C in a final volume of 3 ml containing 100 mmol/l KCl, 25 mmol/l $MgCl_2$, 1.3 mmol/l ADP, 0.11 mmol/l beta-NADH, 81 mmol/l triethanolamine buffer pH 7.5, 1.9 mmol/l 2-phosphoglycerate, 2.3 units/ml pyruvate kinase, and 3.3 units/ml lactate dehydrogenase. The temperature stability of the samples at 50 °C (30–90 mins) was examined in preliminary experiments, because neuron specific enolase is much more resistant to heat-inactivation than non-neuronal enolase (Marangos and Schmechel, 1987). Activity was expressed as nmoles of 2-phosphoglycerate converted/mg of protein/min (nmol/mg/min).

Binding of ^{3}H-QNB to mouse brain. Binding to muscarinic acetylcholine receptors in brain tissues was assessed with ^{3}H-QNB. The binding reaction was terminated by using a centrifugation technique

modified from Birdsall *et al.* (1978). Pellets of brain tissues were suspended in 50 mmol/l sodium phosphate buffer pH 7.4. Five to 8 concentrations (0.2–6.5 nmol/l) of ^{3}H-QNB were incubated with samples containing 75 μg protein at 37 °C for 60 mins. All assays were performed in duplicate. Nonspecific ^{3}H-QNB binding was assessed in the presence of 1 μmol/l atropine sulfate. After the incubation the tubes were centrifuged at 16 000 x g for 90 secs. The pellets were washed in 50 mmol/l sodium phosphate buffer pH 7.4 and incubated overnight in 50 μl Protosol (DuPont Canada). The radioactivity was determined by mixing in scintillation fluid, maintaining at room temperature for at least 1 hr, and counting by liquid scintillation spectrometry.

Analysis of results. Binding data from the saturation experiments were analyzed by computerized linear regression analysis (SigmaPlot; Jandel Scientific, San Rafael, CA) to determine K_D and B_{max} values. The statistical significance of differences between normal and infected brain tissues for both enzyme activities and binding data was determined using Student's unpaired t test.

Results

Enzyme activities

The activities of ChAT, AChE, and enolase were determined in brain homogenates from the cerebral cortex and hippocampus of moribund CVS-infected and uninfected mice (Table 1). Preliminary experiments indicated that the majority of the enolase activity in mouse brains was heat-stable, confirming a preponderance of neuron specific enolase. CVS-infected brains showed only mild reductions in activities of cholinergic enzymes in the cerebral cortex and hippocampus versus controls that were not significant. In contrast, there was slightly greater enolase activity in these regions in CVS-infected brains than controls, which was also not statistically significant.

Table 1. Enzyme activities in mouse brains

Enzyme	Uninfected*	CVS-infected*	Change	p
ChAT (pmol/mg/min)				
Cerebral cortex	5436 ± 294 (n = 13)	4881 ± 288 (n = 13)	-10.2 %	0.19
Hippocampus	7480 ± 512 (n = 12)	6659 ± 271 (n = 14)	-11.0 %	0.15
AChE (nmol/mg/min)				
Cerebral cortex	37.2 ± 1.0 (n = 13)	35.2 ± 1.3 (n = 14)	-5.4 %	0.24
Hippocampus	50.0 ± 2.1 (n = 22)	46.3 ± 1.4 (n = 24)	-7.4 %	0.14
Enolase (nmol/mg/min)				
Cerebral cortex	169.3 ± 7.9 (n = 14)	172.5 ± 5.3 (n = 16)	+1.9 %	0.73
Hippocampus	126.1 ± 5.0 (n = 9)	132.1 ± 5.3 (n = 10)	+4.8 %	0.43

*Results are expressed as mean ± SEM, and n is the number of mice assayed.

Table 2. ^{3}H-QNB binding in mouse brains

Brain region	B_{max} (fmol/mg protein)*				K_D (nmol/l)*			
	Uninfected	CVS-infected	Change	p	Uninfected	CVS-infected	Change	p
Cerebral cortex	2289±82(n = 10)	2125±85(n = 10)	-7.2 %	0.18	0.0583±0.0115(n = 10)	0.0585±0.0113(n = 10)	+0.3 %	0.99
Hippocampus	1958±94(n = 7)	1764±86(n = 8)	-9.9 %	0.15	0.0491±0.0070(n = 7)	0.0448±0.0054(n = 8)	-8.8 %	0.63

*Results are expressed as mean ± SEM, and n is the number of mice assayed.

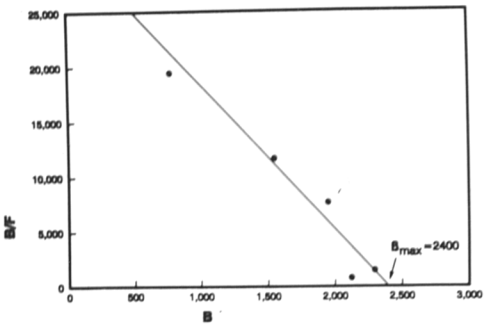

Fig. 1
Scatchard plot of the saturation binding data of ^{3}H-QNB in the cerebral cortex of a CVS-infected mouse
B (fmol/mg) represents the amount of bound radioligand, and B/F [(fmol/mg)/(mmol/l)] represents the ratio of bound to free radioligand. The maximum number of binding sites (B_{max}) is the x-intercept of the line of best fit determined using linear regression analysis. The equilibrium binding constant (K_D) is the negative reciprocal of the slope of the line.

Binding of ^{3}H-QNB in mouse brain

^{3}H-QNB was found to bind specifically to brain membranes from the cerebral cortex and hippocampus. Scatchard analysis was performed with plots of bound/free vs. bound radioligand (Rosenthal, 1967; Hrdina, 1986) (Fig. 1). Binding in both the cerebral cortex and hippocampus of uninfected mice was of high capacity ($B_{max} = 2289 \pm 82$ and 1958 ± 94 fmol/mg, respectively) and high affinity ($K_D = 0.0583 \pm 0.0115$ and 0.0491 ± 0.0070 nmol/l, respectively) (Table 2), and they were similar to values obtained in previous studies in mice (Waller and London, 1983; Kitamura *et al.*, 1989). Although there were slight reductions in B_{max} and K_D values in CVS-infected brains (except for the K_D in cerebral cortex), these changes were not statistically significant.

Discussion

Defective cholinergic synaptic neurotransmission is a possible explanation for the neuronal dysfunction that is observed in rabies. The cholinergic system has wide projections in the brain, and Tsiang (1982) found decreased ^{3}H-QNB binding to muscarinic acetylcholine receptors in brain regions of CVS-infected rats. In addition, Lentz and coworkers (Lentz *et al.*, 1982; 1985; 1986; Lentz, 1985) have provided evidence that rabies virus binds to nicotinic acetylcholine receptors at neuromuscular junctions, which might play a role in rabies pathogenesis. Hanham *et al.* (1993) have also recently reported reduced IFA staining of pyramidal neurons in the hippocampus of moribund CVS-infected mice using an anti-idiotypic monoclonal antibody that reacts with the nicotinic acetylcholine receptor.

Widespread infection of the brain develops after peripheral inoculation of mice with CVS (Jackson and Reimer, 1989). If there is defective cholinergic neurotransmission in this experimental model of rabies, then one might observe significant differences in either the activities of ChAT and AChE or in binding to

muscarinic acetylcholine receptors in the cerebral cortex and/or hippocampus of moribund CVS-infected and uninfected mice. A previous study has shown that these brain regions contained large number of rabies virus-infected neurons by using immunoperoxidase staining (Jackson and Reimer, 1989). Although there were very mild reductions (5–11 %) in activities of both ChAT and AChE in these regions compared to uninfected controls, they were not statistically significant. Similarly, ^{3}H-QNB binding in the cerebral cortex and hippocampus of CVS-infected mice showed only small differences that were not statistically significant.

Since these studies were performed on moribund CVS-infected mice, significant alterations of cholinergic markers should have occurred if they had played an important pathogenetic role in producing the severe clinical neurological illness. Dysfunction of the cholinergic system in rabies that is selective for other regions of the central nervous system or for other aspects of synaptic neurotransmission (e. g. release of acetylcholine and binding to nicotinic acetylcholine receptors) was not studied. The large reductions in ^{3}H-QNB binding in the cerebral cortex and hippocampus of CVS-infected rats reported by Tsiang (1982) were not confirmed in this study in mice using peripheral inoculation. This could be due to a difference in the species, route of inoculation, or methodologies. Scatchard plots provide a superior analysis of binding data (Rosenthal, 1967; Hrdina, 1986).

The basis for the neuronal dysfunction in rabies is unknown. Defective synaptic neurotransmission remains an attractive hypothesis to explain the neuronal dysfunction. The present study does not provide supporting evidence that dysfunction of the cholinergic system in the brain is of fundamental importance in the pathogenesis of rabies. The search for the role of other neurotransmitters and excitatory amino acids (Lockhart *et al.*, 1991) in rabies should continue.

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