Tracking the footprints of the rabies virus: are we any closer to decoding this elusive virus?

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Running title: Biology of rabies viral encephalitis

Key words: rabies, neurovirulence, apoptosis, Negri bodies, glycoprotein

Word count: 5806

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Competing interests - None
Abstract

Rabies viral encephalitis, though one of the oldest recognized infectious disease remains an incurable, fatal encephalomyelitis despite significant advances in understanding of its pathobiology. Advances in science has led us to the trail of the virus in the host, but the sanctuaries in which the virus remains hidden or “latent” for its survival is unknown. Insights into host-pathogen interactions has facilitated evolving immunologic therapeutic strategies but we are far from finding a cure. To explain dichotomy in clinical presentation and uniform mortality in this viral encephalitis, various factors have been implicated that include both viral and host factors such as neuroanatomical distribution of viral antigen within brain, alteration in host immunity or viral strain in the former whereas apoptosis, neuronal dysfunction and neurotransmitter abnormalities in critical neuroanatomical areas are popular explanations for the latter. Most of the present day knowledge has however evolved from *invitro* studies using fixed (attenuated) laboratory strains that may not be applicable in the clinical setting. Much has to be learnt about this elusive virus before effective therapeutic strategies can be evolved. This review attempts to address some of these questions that have remain unanswered in light of knowledge available from recent advances.
Introduction

Rabies is one of the oldest and most feared human diseases known to man that continues to be a major public health problem in developing countries. It is an acute, progressive, incurable fatal encephalitis, caused by a highly neurotropic RNA virus, taxonomically classified in the genus lyssa virus, family Rhabdoviridae. Mammalian reservoirs for this exclusively neurotropic virus include Carnivora and Chiroptora, but transmission by rabid dogs still pose greatest health hazard worldwide. Despite the fact that all warm blooded animals are susceptible, rabies virus in any given zoonotic area adapts to a single dominant reservoir host. For instance, in India, dogs are the main vectors, accounting for 95% of the reported cases. In the United States of America, striped skunks, raccoons, foxes, and coyotes are the most important reservoirs perpetuating wildlife rabies in contrast to foxes and raccoons in Europe, and dogs and mongooses in South Africa. Bat rabies that cause significant threat in America and Europe, is not reported from India. Human to human transmission has been recorded through corneal, liver and kidney transplants taken from donors with rabies misdiagnosed as Guillain Barre syndrome or stroke.[1,2,3]

Despite continued attempts at medical intervention, rabies remains one of the infectious diseases with the highest case fatality ratio.[4] The disease has no discrimination for age, gender, geography, or occupation. According to WHO estimates, 50,000 human deaths are reported world wide every year. The majority are from developing countries of Asia and Africa[5] 60% of the cases being reported from India alone [6] Human rabies continues to be endemic in India except for islands of Andaman, Nicobar and Lakshadweep.[7]

Rabies, one of the oldest recognized infectious disease has a rich and fanciful history. The dramatic and extraordinary clinical manifestations with tendency to violence or rage forms the basis for the name for this dreaded disease in various languages; “rabere” (to rage or to rave) in Latin, “rabhas”(Violence) in Sanskrit and “lyssa” or “lytta”, which means “madness’” in Greek.

Two distinct clinical syndromes – furious and paralytic rabies are recognized in humans. Historically the former, dominated by limbic symptoms was the most recognized form of the disease with prototypic symptoms of hydrophobia, aerophobia and aggressive behaviour. The paralytic form, on the other hand presenting with ascending paralysis without developing “hydrophobic” symptoms was initially recorded by Gamaleia in 1887,[8] but was not widely recognized until several decades later.[9-11] More recently recognized is the bat-related rabies with clinical features substantially different from those of dog-related rabies. [4] It is conventional to refer to the virulent form of rabies virus in nature and in infected animals as “street” (or “wild-type”) virus and the laboratory passaged (avirulent) form as “fixed” virus (Challenge Virus Standard strain - CVS). This connotation has been followed in this presentation

Rabies remains an important public health problem in many developing countries long after effective vaccination strategies have virtually eradicated other dreaded diseases such as small pox, diphtheria, polio and cholera.
Efficacious rabies vaccines have been around for a long time and combined with stray animal control measures, public health infrastructures, and good clinical evaluation of exposure, the risks posed by terrestrial animals can be minimized. However effective treatment of the disease remains elusive. Despite almost yearly updates on this disease,[12-14] we are far from understanding the pathogenesis of this enigmatic disease. Most of our present knowledge comes from studies on experimental animals, mostly rodents, infected with fixed (attenuated) laboratory strains of the rabies virus that do not truly reflect natural infection with street virus (virulent) strains causing infection in humans or animals. A number of critical questions such as dichotomy in clinical manifestations, highly variable incubation period, and cause for uniform fatality remain unanswered. In this review, some of these aspects are addressed with a relook at present state of knowledge, with a hope that a better understanding of the pathogenesis will help in evolving effective therapy for this fatal disease.

**Question 1: Extreme variability in incubation period (IP): what must a virus do to remain concealed for such a long time?**

The most common mode of infection is by introduction of virus-laden saliva into the victim following bite of a rabid animal. Clinical disease does not usually develop for a period of weeks to months and at times years. The incubation period is therefore most variable, ranging from 6 days to as long as 6 years akin to a slow virus disease.[15,16]

Both the host and viral factors have been implicated to explain the extreme variability in IP. The age of the host, degree of neural innervation of the inoculation site (site of bite), distance between the point of inoculation to spinal cord or brain, viral strain and quantum of virus introduced, postexposure treatment and yet unknown factors influence the IP.

**HOST FACTORS**

1. **Site of entry**

During early incubation period (IP), the virus remains sequestered at the site of inoculation and quickly enters an eclipse phase, and remains undetectable by presently known techniques. The precise location and structure of the virus during this period remains amongst the most puzzling and intriguing aspect of rabies pathogenesis. In cases with longer incubation period, there is limited evidence that suggests that the virus remains close to the site of inoculation. The local replication time could influence the interval between exposure and clinical signs (Fig.1A).[17] Evidence for viral replication in muscle cells, in skunks intramuscularly inoculated with wild-type virus, has been demonstrated by RT-PCR and immunohistochemistry. [17] Following local replication in the muscle cells, the virus enters unmyelinated nerve endings at the neuromuscular junctions or the muscle spindles (Fig.1B). Replication might be necessary to generate sufficient quanta of viral copies for entry into the peripheral nervous system (Fig.1C-E).[18,19] Direct entry into peripheral nerve without a replicative cycle in extraneural cells has occasionally been recorded.[20-25] Rodent models inoculated with fixed rabies strain, the IP is short.[26,27] In cases with multiple
bites in the head and neck region, direct viral entry without local replication may be responsible for very short IP. Alternatively, high titres of antirabies antibodies acquired by active or passive immunization could restrict viral replication and protect against infection thereby influencing IP whereas effective cell mediated immunity is essential for eventual elimination of the virus.

2. Receptors for entry and transport

In the peripheral nervous system, the virus binds to nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction.[28] Two additional putative receptors have been reported – neural cell adhesion molecule (NCAM) expressed in the adult muscle and neuromuscular junction and the p75 neurotrophin receptor.[29-32]

**NACH receptor:** The nACh receptor was the first identified for viral entry at neuromuscular junctions. α-bungarotoxin, an antagonist of the nACh receptor provided partial inhibition of infection in cultured chick myotubes and rat myotubes.[28] However, its importance in the CNS is unclear as neurons and non-neuronal cells not expressing nAChR are also highly susceptible to rabies viral infection. Variations in animal susceptibility to rabies have been recognized for many years. For instance, foxes are highly sensitive, dogs less sensitive and opossums are highly resistant to rabies infection. The differential susceptibility could reflect the density and distribution of nAChR in their muscles and other peripheral organs.[33,34]

**Neural Cell Adhesion Molecule (NCAM):** By *in vitro* tissue culture studies, susceptible cell lines were found to have NCAM molecule on their surface and treatment with heparan sulphate (a ligand for NCAM) or with anti-NCAM antibodies, significantly reduced the susceptibility. The *in vivo* relevance of the NCAM as a receptor for rabies virus was demonstrated in NCAM-deficient mice. Mortality was delayed and brain invasion by the virus was drastically restricted in these mice, indicating an important role for NCAM in rabies virus infection and spread.[29]

**Low-affinity p75 Neurotrophin Receptor (p75NTR):** Role of p75NTR, as a receptor for “wild type” rabies virus was first suggested by Tuffereau et.al. However in a recent study, Langevin and colleagues reported that the rabies virus glycoprotein has a high affinity ligand for a non-neurotrophin site on p75.[32,35] These findings raise questions about the importance of the p75NTR in the pathogenesis of rabies, although it is possible that the repertoire of receptors used by the street and fixed rabies virus strains are completely different *in vivo*.

**Other cell surface molecules:** Neuraminidase and β-galactosidase treatment of cells had an inhibitory effect on virus binding, α-mannosidase had an intermediary effect and fucosidase had no effect.[36,37] Lectins were shown to mediate the inhibition of rabies virus binding to cells.[37] Cellular sialic acid, galactose, mannose and N-acetylglucosamine, gangliosides [37] but not fucose were shown to be involved in the binding of rabies virus to the host cells. Variability in receptors used for viral entry could influence the IP by altering the route taken to reach the CNS and the neuronal groups involved.
3. Pathway of spread to CNS: does the route taken by the virus influence the incubation period?

Neuroanatomical distribution of rabies virus in various brain nuclear areas following peripheral inoculation has demonstrated that the virus propagates in the CNS across chains of synaptically connected neurons. In fact, the virus has been used as a neuroanatomical tracer to define neuronal circuits in rodents and primates.[38-41] These studies have provided strong evidence that fast axonal transport of the virus occurs in a retrograde fashion.[42,43] Travel to the CNS via peripheral axons occurs at a fairly constant rate of 12-24mm per day.[39,44,45] Colchicine, a microtubule disrupting agent that effectively inhibits fast axonal transport, prevents viral spread confirming the hypothesis.[46]

Although all available data supports centripetal spread of rabies virus by axonal transport from the site of inoculation to the CNS,[47] mode of virus spread within the CNS appears to be more complex. At present, three potential pathways are postulated: (1) virus dissemination within extracellular spaces, (2) intraaxonal transport via by fast axonal transport, and (3) cell-to-cell transmission between contiguous cells and their processes.

Once released into extracellular spaces, wide dissemination in extracellular spaces and the CSF pathways is an efficient and rapid mechanism of virus dissemination within the CNS. In fact, the width of intercellular spaces in the host nervous system is sufficient to allow transit of the virus.[48] but this route plays a major role in rapid progression of fixed strain of the virus as budding from the cell surface precedes the intracellular maturation (Fig. 2A, B).

Transit by axonal route, on the other hand, is more important in the slow progression of street virus infection. Virions and matrices are observed within the dendrites and axons of the nervous system as frequency of virus budding from the cell surface is lower compared to fixed virus infection. Transit of the virus within the axon also allows it to circumvent the host immune responses and infection can progress without recognition by the host immune system. Importance of long ascending and descending tracts as a route of virus spread within the CNS was emphasized in an immunohistochemical study on skunks.[49] Cell-to-cell transmission of virus between contiguous cells and their processes particularly at synaptic junctions has been observed frequently, in experimental animals and less frequently in human rabies.[18,50,51] This type of transit not only facilitates virus spread between adjacent cells but is also important in transsynaptic transmission of the virus from distant sites playing a decisive role in the entry into CNS.[18]

Although the possibility of intra axonal transport along nerves was first postulated by Morgagni in 1769, it was almost a century later that Pasteur and Roux demonstrated presence of infectious particles in nervous tissues of rabid animals.[52] Experimental studies conducted by Baer et al indicated that though perineurial cells and myelin were not involved in viral transport, interruption of axoplasmic flow by alkaloid drugs such as colchicines and vinblastine succeeded in preventing the ascent from the site of inoculation to CNS.[46,53,54] The virus further replicates in the dorsal root ganglion (sensory neuron) and anterior horn cells (motor neurons).[55] Discrimination between sensory and motor nerves as
pathways for rabies virus to access the CNS has always been a matter of controversy. Johnson found simultaneous presence of virus in sensory and motor neurons.[23] Schneider held that sensory nerves are the main routes of infection as evidenced by frequent presence of viral inclusions in dorsal root ganglia.[56] Anterograde transport may occur either passively through cell body without replication, to be released from neuritic ending or by budding from the cell surface.[57] The exact morphological form of virus during passive axoplasmic flow, whether as mature virion or a ribonucleoprotein complex, is unclear but the integrity of the neuronal microtubule network remains vital.[46,58,59]

The route taken by the virus to reach the CNS may dictate the length of IP (Fig.3). For instance, direct access to brain stem with involvement of critical areas such as respiratory and vasomotor centers in medulla oblongata may lead to rapid evolution of disease process and death with short IP. On the contrary, involvement of long ascending tracts to reach the cortical areas or sequestration of the virus in peripheral ganglionic areas may lead to long IP. Subsequent reactivation by factors yet unknown and mobilization of virus to reach critical areas in brain may occur terminally and herald progression along neuroanatomical pathways leading to fatality.

VIRAL FACTORS

Several studies emphasize the importance of the rabies virus glycoprotein in the uptake, transport, transsynaptic spread, and topographic distribution in the nervous system.[60-62] Two recent studies provided evidence that the rabies virus phosphoprotein, particularly amino acid residues at positions 143 and 147 interacts strongly with 10-kDa cytoplasmic dynein light chain (LC8).[63-65] LC8 is important in microtubule-directed organelle transport and actin-based vesicle transport, facilitating axonal spread. Neuroattenuation of virus was demonstrated experimentally in mice inoculated with mutant virus with deletion in phosphoprotein encompassing LC8-interacting motif and simultaneous substitution of arginine at position 333 of viral glycoprotein but not with deletion of LC8 alone.[66]

Although a number of factors are implicated, it still remains to be elucidated which of these factors – virus strain and concentration, inoculation route, genetic susceptibility or host factors, either singly or in combination modulate the variable incubation period in different hosts.

Question 2: What is responsible for dichotomy in clinical presentation?

Clinically the disease presents in two distinct forms - furious (encephalitic) or paralytic (dumb) rabies. Two thirds of patients develop furious form while the remaining present with paralytic form of rabies.[15,67] The former is characterized by periods of agitation and confusion alternating with period of lucidity, aerophobia, hydrophobia and signs of autonomic dysfunction like lacrimation, pupillary dilatation, hypersalivation and excessive sweating. Paresthesias and weakness marks the onset of the paralytic form in the bitten extremity progressively involving all the limbs, pharyngeal and respiratory muscles. This form causes diagnostic dilemma as aerophobia and hydrophobia
are present in only half of such cases, that too terminally before the stage of coma. Furthermore, early in the course of the disease, flaccid paralysis of all the limbs and areflexia, closely resembles Landry’s Guillain-Barre syndrome (GBS), with relative sparing of consciousness. This problem is further compounded when the history of dog bite is not forthcoming. Clinical course is generally prolonged to weeks and paralytic features predominate. The pathogenetic basis for the two different clinical forms of rabies has always been a subject of intense interest. A number of hypothesis have been proposed.

**Does neuroanatomical distribution of rabies viral antigen determine clinical presentation?**

Neural pathway of spread of rabies virus is now well established and demonstrated in experimental models.[47] The exact pathway of viral dissemination is not known once the virus reaches the central nervous system.[49,68]

Clinical signs and symptomatology of paralytic and encephalitic forms of rabies suggest involvement of spinal cord and peripheral nerve in the former and cerebral cortex and limbic system in the latter.

However, Tirawatnpong et al found no significant differences in regional distribution of rabies virus antigen or inflammatory changes between furious and paralytic forms on autopsy studies.[69] Brain stem and spinal cord were preferential sites of involvement as determined by immunohistochemistry, regardless of clinical type, when survival was less than 7 days. By eight days after the clinical onset, the antigen was found disseminated throughout the neuraxis. However, as the authors are careful to point out, it is possible that terminally, the distribution of the virus in the brain could be much more widespread and may not reflect the distribution at the time of the patient’s presentation.

Histomorphological studies in paralytic rabies demonstrated prominent inflammation, and widespread neuronal destruction in brain stem and spinal cord in comparison to cerebral cortex. But immunohistochemical studies found no correlation between the anatomical distribution of rabies viral antigen and clinical manifestations.[11,15]

Antemortem magnetic resonance imaging (MRI) has also shown predilection for brain stem, hippocampus and hypothalamus involvement in both clinical forms.[70] MR imaging in five cases (2 furious and 3 paralytic) demonstrated ill defined hyperintensities on T2 weighted images in brain stem, hippocampi, hypothalamus, subcortical and deep white matter and cortical grey matter as early as day 3 of onset even when consciousness was preserved. Contrast enhanced lesions were seen in brain stem, hypothalami and spinal nerve roots only when patients became comatose. The anterior horn of spinal cord was involved in both forms on gradient echo T2 weighted images. Hence neither the distribution of viral antigen nor MRI changes could explain evolution of clinical symptomatology.

Clinical, electrophysiological and pathological evidence suggests that peripheral nerve dysfunction contributes to weakness in paralytic form.[11,71] On
electrophysiological studies, multifocal demyelination was noted in all the cases of paralytic rabies.[71] In contrast, abundant denervation potentials with normal sensory and motor nerve conductions suggesting anterior horn cell involvement was evident in the bitten limb in cases with encephalitic form, though demonstrable weakness was not found. Pathological studies confirmed presence of peripheral nerve inflammation and demyelination as the prime pathological manifestation in cases of paralytic rabies and chromatolysis in anterior horn cells in encephalitic form. [11,71,72] The striking similarity in clinical as well as pathological changes between paralytic form of rabies and Guillain-Barre syndrome (GB Syndrome) raised the possibility that the same immunopathogenetic mechanism implicated in GB syndrome associated with Campylobacter jejuni, Mycoplasma pneumonia, CMV and EBV could be operative in paralytic rabies as well, with development of autoantibodies to peripheral myelin due to molecular mimicry.[73] In fact one of the initial cases of acute motor sensory variant of GB syndrome on review was found to be a case of paralytic rabies.[74]

**Do viral factors play a role?**

It has been postulated that variants of rabies virus associated with different vectors produce varied clinical manifestations. Hemachuda and co-workers analysed the entire glycoprotein (G), nucleoprotein (N) and phosphoprotein (P) genes of rabies viruses from two cases each of paralytic and encephalitic variants. They did not find any single nucleotide or amino acid specific pattern with either form of rabies infection.[75] The differences found in G and P proteins were outside the ectodomain and not in interactive region of G protein responsible for virus pathogenicity nor did they lie in the immunodominant G domain.

The rabies viral glycoprotein (G) is critical for both neutralizing antibody production and initiation of cell-mediated immune response. Differences in G protein affects G protein-receptor interactions with nicotinic acetylcholine receptor at the site of bite, P75 neurotrophin receptor and anti-glycolipid or ganglioside CNS receptors. Minor variations in G protein such as substitution of arginine at position 333 affects neuroinvasiveness by use of different neuronal pathways.[76] Modification of dynein light chain binding site to viral capsid P protein can reduce efficiency of peripheral spread of the rabies virus.[66]

**Do host factors play a role?**

The differences in clinical symptomatology despite the nearly identical MRI features and neuroanatomical distribution of viral antigen argue against existence of different virus strains and suggest instead participation of host factors in modulating progression and terminal outcome.

The role of cell mediated immunity was suggested by findings of demyelination and inflammation of nerve roots with the cellular infiltrates characterized as T cells.[71] Patients with cell mediated immune response to rabies virus and with high serum concentrations of IL-2 receptor and IL-6 manifest as encephalitis are likely to succumb sooner.[77] In contrast, patients with paralytic rabies have defective immune responsiveness, including lack of
lymphocyte proliferative responses to rabies viral antigen and lower levels of serum cytokines, including interleukin-6 and soluble interleukin-2 receptor. No antibodies to myelin basic protein has been demonstrated.

Molecular mimicry between innate and foreign antigen is well accepted in the pathogenesis of GB syndrome, a condition that closely mimics paralytic rabies. Immune mediated damage to rabies infected axons, most marked in the ventral spinal nerve roots was noted in a case of paralytic rabies, closely mimicking axonal variant of GB syndrome. Participation of autoantibodies against peripheral nerve antigens in the evolution of paralysis is also suggested by vivid enhancement of ventral and dorsal nerve roots on MRI, identical to that seen in classical cases of GB syndrome. Neutralizing antibodies have not been consistently demonstrated in CSF from cases of paralytic rabies. Although no antiganglioside antibodies could be demonstrated, other pathogenic autoantibodies should be sought. The initiation and amplification of cytokine cascade may influence viral modification of host gene expression that inturn plays a role in viral replication, spread and neuronal survival.

**Basis for behavioral changes**

The neuroanatomical basis for the behavioral changes in humans and animals with rabies encephalitis have not yet been well characterized. Aggressive behavior exhibited by natural hosts are not found in experimental animals inoculated with fixed laboratory strain of the virus. But striped skunks inoculated peripherally with virulent wild-type strain of rabies virus exhibited aggressive responses. Histologically, heavy accumulation of viral antigen was found in the mid brain raphe nuclei, red nucleus, dorsal motor nucleus of vagus, and hypoglossal nucleus in skunks infected with street virus in contrast to those inoculated with fixed CVS strain of rabies virus. Impaired serotonin neurotransmission from the raphe nuclei in the brain stem could be responsible for development of aggressive behavior in natural vectors of rabies. Limbic system involvement and dysfunction are long suspected to play an important role in behavioral changes, like loss of natural timidity, aberrant sexual behavior, and aggressiveness. However experimental studies in rodent models have not shown histological alterations in the corresponding neuroanatomical areas and hippocampal infection was actually found relatively late following peripheral routes of inoculation suggesting species variation in pathomorphological evolution. Aggressive behavior is essential for the efficient horizontal transmission of virus to other hosts by biting. The neural mechanisms of aggressive behavior is still poorly understood.

**BRAIN DYSFUNCTION IN RABIES**

Despite the dramatic and severe clinical neurologic signs in rabies, the neuropathologic findings that typify a viral encephalitis (inflammation and neuronophagia) are conspicuously mild. Neuronal dysfunction rather than morphological changes could be playing a more important role. Although the basis for the neuronal dysfunction in rabies at the cellular level is not clear,
current understanding of abnormalities in neurotransmission, electrophysiologic alterations, effects on ion channels and causes of neurotoxicity are reviewed by Fu and Jackson.[86] Dysfunction of ion channels in infected cell culture and induction of inducible nitric oxide synthase mRNA and increased levels of nitric oxide have been demonstrated in rodents inoculated with the virus.[87-89] These observations are however limited to experimental studies and their significance in natural rabies is uncertain.

**Defective Neurotransmission**

**Acetylcholine**

The hypothesis that defective cholinergic neurotransmission might be the basis for neuronal dysfunction in rabies, led to the investigation of specific binding to muscarinic acetylcholine receptors. Experimental results varied depending on species (mouse versus rat) and the route of inoculation (peripheral versus intracerebral). The neuroanatomical viral antigen distribution appears to correspond to cholinergic areas in the mouse brain suggesting a strong association in pathogenesis (Fig.4).

In rats inoculated with CVS strain of rabies virus, the greatest reduction in binding was noted in the hippocampus but less in the cerebral cortex and caudate nucleus.[90] However in mice infected with the same virus, no difference in binding was seen between cerebral cortex and hippocampus nor in enzymatic activities of choline acetyl transferase and acetyl transferase, required for synthesis and degradation of acetylcholine, respectively.[91]

In naturally infected rabid dogs with either furious or dumb rabies, reduced binding affinity of muscarinic acetylcholine receptors was noted in hippocampus and brainstem, but not in other brain regions though receptor content remained unchanged.[92] The reduced receptor affinity was independent of the presence or absence of viral antigen at that site suggesting that perturbation of receptor binding was not dependent on clinical manifestations or the local viral load.

**Serotonin**

Serotonin has a wide distribution in the brain, and is important in the control of sleep and wakefulness, pain perception, memory, and behavior.[93] Ligand binding to serotonin (5-HT) receptor subtypes was studied in the brains of animals infected with CVS strain of virus.[94] Serotonin binding was not affected in the hippocampus, but decreased markedly in the cerebral cortex 5 days postinoculation suggesting that rabies virus specifically targets 5-HT1D-like receptors in the cerebral cortex. Reduced binding was demonstrated before rabies virus antigen was detectable in the cerebral cortex suggesting that receptor binding is not related to viral replication in cortical neurons but could be stress induced. There are important serotoninergic projections from the dorsal raphe nuclei in the brainstem to the cerebral cortex and this can facilitate early infection of the midbrain raphe nuclei in experimental animals.[83] Decreased potassium evoked release of [3H] 5-HT synaptosomes from the cerebral cortex of rats infected with CVS strain of rabies virus was found compared with controls.[95] Hence impaired release and binding of serotonin, might play an important role in the neuronal dysfunction in rabies.

**γ-Amino-n-Butyric Acid**
Impairment of both release and uptake of $\gamma$-amino-n-butyric acid (GABA) have been found in primary rat cortical neuronal cultures infected with CVS strain of virus.[96] A 45% reduction of [3H] GABA uptake was found 3 days after the infection, coinciding with the time of peak viral growth in cultures. The importance of these abnormalities in both the uptake and release of GABA on rabies pathogenesis \textit{in vivo} is yet to be determined.

\textbf{Electro physiologic Alterations}

Electroencephalographic (EEG) recordings of mice infected with CVS strain of rabies virus - showed that the initial changes were alterations in stages of sleep cycle, including the disappearance of rapid-eye-movement (REM) sleep and development of pseudoperiodic facial myoclonus.[97] Later, a generalized EEG slowing (at 2-4 cycles per second), and terminal extinction of hippocampal and cortical activity was recorded. Brain electrical activity terminated about 30 minutes before cardiac arrest, indicating that in experimental rabies cerebral death occurs prior to failure of vegetative functions. Street virus-infected mice showed progressive disappearance of all sleep stages with a concomitant increase in the duration of wakeful stages (indicating insomnia). These changes occurred before the development of clinical signs of rabies.[98] There was a curious absence of EEG abnormalities in street virus-infected mice lasting through preagonal phase of the disease. Pathomorphological changes are more marked in neurons infected with fixed rabies viruses than wild type strains, consistent with the idea that functional/physiological impairment of neurons is much more important in natural wild type rabies than in infection with fixed virus stains.

\textbf{Ion Channels}

Effect of viral infection on ion channels of neurons was studied \textit{invitro} using rabies virus (RC-HL strain) infected neuroblastoma cells and whole cell patch clamp technique.[87] Infection reduced functional expression of voltage-dependent sodium channels and inward rectifier potassium channels, leading to decreased resting membrane potential. Reduction in sodium channel and inward rectifier potassium channels could prevent infected neurons from firing action potentials and generating synaptic potentials, resulting in functional impairment. No functional alteration of voltage-dependent calcium ion channels was demonstrable \textit{invitro].[99] $\alpha_2$-adrenoreceptor-mediated inhibition of voltage-dependent calcium ion flux is demonstrable which serves as a brake to keep neurons from releasing their neurotransmitters beyond physiologic requirements. The impaired modulation by $\alpha_2$-adrenoreceptors could contribute to hyperexcitability and aggressive behavior in rabies.[99]

\textbf{Nitric Oxide}

The short-lived gaseous radical nitric oxide acts as a biologic mediator for diverse cell types. Produced by many different cells, it mediates a variety of functions, including vasodilation, neurotransmission, synaptic plasticity in the brain, neurotoxicity and immune cytotoxicity.[100,101] Nitric oxide is released by the enzyme nitric oxide synthase (NOS), which also produces other reactive oxides of nitrogen.[100]
A protective role in the pathogenesis of viral infection was suggested by the observation that inhibition of NOS results in increased mortality of infected mice. [102] Alternatively, induction of iNOS mRNA occurring in mice infected experimentally with wild type rabies virus suggested that nitric oxide and/or other endogenous neurotoxins may mediate neuronal dysfunction in rabies and other infectious diseases.[88,103] Progression of the disease also correlated with increasing quantities of nitric oxide in the brain to levels up to 30 fold more than in controls.[89] Further, treatment with iNOS inhibitor aminoguanidine (AG) delayed the death of CVS strain of rabies infected mice.[104] The role of nitric oxide in rabies pathogenesis remains to be determined because it exerts both beneficial and detrimental effects.

DEGENERATION OF NEURONS

In natural rabies, pyknotic chromatolytic neurons are seen throughout the CNS most frequently in brainstem, and periaqueductal gray matter, cervical spinal cord, thalamus and less frequently the cerebral cortex.[105] In general, these degenerated neurons do not harbor Negri bodies but are positive for viral antigens and may be accompanied by inflammatory reaction.[51]

In comparison to natural infection by a “wild type” virus, fixed rabies virus infection exert more severe cytopathic effect in both cell cultures and animals. The descriptions of chromatin condensation in fixed virus-infected neurons could be a reflection of apoptosis. Apoptosis may occur with or without viral antigen expression, and the mechanism of apoptosis maybe related directly or indirectly to virus replication. Apoptosis in Bax-deficient mice was less severe in the cerebral cortex, hippocampus, and cerebellum than in wild-type mice, but moderate to severe in the brainstem in both strains of mice.[106] The regional difference in severity of neuronal degeneration and selective cellular vulnerability in rabies infection can be explained in part by the inducibility of “Bax protein”, a pro-apoptotic protein, in the host cells.[106]

INFLAMMATORY REACTIONS

Perivascular and parenchymal collections of inflammatory cells once thought to be specific for rabies, are neither unique nor specific. Perivascular infiltrates are composed of lymphocytes and monocytes intermingled with small numbers of polymorphonuclear and plasma cells, depending on the stage and severity of infection. The phenotype of these inflammatory cells is not well studied. Iwasaki et al in one case of human rabies, showed 50-70% of mononuclear cells in the perivascular spaces to be CD3-positive T-lymphocytes, more than one-third among these being CD4 helper T-lymphocytes. CD20-positive B-lymphocytes were negligible, and the remaining were CD68-positive cells of monocyte/macrophage lineage cells.[107]

As in other viral encephalitides, neuronophagia and glial nodules, are seen often in areas where neuronal degeneration and inflammatory cell infiltration are conspicuous (Fig.5A-E). Topographic dissociation between location of inflammatory reactions and of Negri body has been reported in an autopsy study of 49 cases. Inflammatory changes were most frequently found in
medulla, pons and spinal cord followed by thalamus and less frequently in cerebellum and hippocampus. Negri body bearing neurons were rare where inflammation was dense.[105]

**Question 3: What is cause of death in rabies viral encephalitis?**

Under natural conditions, rabies virus infection causes relatively mild neuropathological changes without significant evidence of neuronal death.[108] Based on these observations, possibility of neuronal dysfunction rather than death was suspected to explain neurological symptoms in rabies.

Cell death by apoptosis or necrosis has been implicated in a number of viral diseases. [109,110] Early reports suggested that apoptosis plays an important role in causing cell death in animals inoculated with laboratory adapted CVS strain of rabies virus.[111] Intracerebrally inoculated rabies virus (CVS strain) caused strong apoptosis in cerebral cortex and hippocampus in suckling and adult mice in contrast to peripherally inoculated animals.[112-114] A correlation between neuroanatomical distribution of apoptotic cells and viral antigen load was observed.[41,112,115]

Different neuronal cell types however appear to have differing susceptibility to apoptosis as cerebellar Purkinje cells and embryonic spinal motor neurons in mice were resistant to apoptotic cell death.[112,116] Viral strains also appear to be important as more neurovirulent forms produce less apoptosis and vice versa both in vitro and in vivo.[113,117] These findings suggest an inverse relation between pathogenicity and apoptosis. Induction of apoptosis may be a host defense mechanism to restrict viral spread and severity of neurological disease. Use of minocycline, an antiapoptotic and anti inflammatory agent in rabies infected mice showed early onset of neurologic signs and greater numbers of infected neurons with high mortality.[118] The findings in experimental animals or in vitro studies with laboratory strains of the virus may not accurately reflect what happens in natural infection in vivo, as conflicting results are reported following intracerebral inoculation of wild type virus in mice. Ubol and Kassisith reported massive apoptosis in the brains of swiss albino mice inoculated with bat strain of rabies virus [119,]. However Yan and co-workers found minimal apoptosis in mice inoculated with silver haired bat rabies virus strain.[120] Factors other than viral strains may play a role in induction of apoptosis.

The level of G protein expression has been correlated with the induction of apoptosis in vitro and in vivo.[113,120] Jackson et al showed morphological changes of apoptosis in pyramidal neurons of hippocampus and cerebral cortex in particular, though it is unclear if the degree of apoptosis varied from region to region within the infected brain.[112] This may have a bearing on severity of clinical symptoms progressing to death. Juntrakul and colleagues in fact implicate delayed cell death of motor neurons in spinal cord and reticular activating system to explain absence of weakness and spinal cord dysfunction despite presence of rabies viral antigen.[121] Park and colleagues demonstrated the absence of apoptosis in dorsal root ganglia, spinal motor and sensory neurons despite presence of viral antigen.[115]
In a recent study, transgenic mice expressing yellow fluorescent protein were inoculated with fixed virus used to study different neuronal populations. A prominent finding was prominent neuronal vacuolation of the cell soma, dendrites and presynaptic endings in cerebral cortical and hippocampal neurons probably related to mitochondrial damage. Apoptosis was strikingly minimal. The authors suggested that structural changes and organelle failure was more important than apoptosis for the fatal outcome in rabies viral encephalitis.[122] In natural rabies, whether neuronal death is by apoptosis or mitochondrial dysfunction remains to be elucidated. Complex mechanisms in synergy may be operative in deciding cell death or survival *invivo* as till date only a single report documents rabies-induced apoptosis.[123]

Significant advances have been made in determining the pathobiology of this viral infection, but a number of questions still remain unanswered, not the very least the neuroanatomical and pathophysiological basis for the characteristic “hydrophobic” symptoms unique to this viral infection that is curiously absent in animal models. The mechanisms operative for its exclusive neurotropism, successful immune evasion and almost fatal outcome without causing evident morphological alterations remains an enigma. The greatest stumbling block has been that despite animal models being available, the findings seen *invitro* with laboratory strains of virus are not reflected *invivo* with wild type strain. Until the modus operandi of the elusive rabies virus is decoded, treatment of this fatal encephalomyelitis will remain a distant dream.
**Legends:**

**Fig.1:** Panel representing events at site of entry of rabies virus.
A: From a patient with rabies encephalitis, skeletal muscle taken from site of bite shows intracytoplasmic eosinophilic inclusion (arrows) representing the Negri body. HEx320
B: Immunohistochemical labeling of motor end plate (arrows) at neuromuscular junction from site of intramuscular inoculation of CVS strain of rabies virus into mouse thigh muscle. Immunoperoxidase for rabies viral nucleocapsidx120
C: Electron micrograph of sciatic nerve showing intraaxonal rabies viral particles in various morphological forms. X27,000
D: Intraneuronal Negri bodies within dorsal root ganglion cells of mouse. HEX320
E: Spread of rabies virus along the dorsal nerve roots with involvement of dorsal root ganglia (arrows) and the spinal cord motor and sensory neurons diffusely. Immunoperoxidase for rabies viral nucleocapsidx12

**Fig.2:**
A: Cell lines (Neu2A) infected with CVS strain of rabies virus demonstrates fluorescent rabies viral antigen aggregated along the cell membrane representing budding from the cell surface. ImmunofluorescenceX400
B: Electron micrograph shows viral particles budding along the cell membrane into the extracellular space of a brain stem neuron in mice inoculated with CVS strain of rabies virus. X36,000

**Fig.3:** Conceptual diagram tracing the different routes of rabies viral entry from periphery to the central nervous system to explain the variability in incubation period. Direct access to brain stem may result in short incubation period (represented by straight arrows). In contrast, entry into cerebellum and cortical areas prior to reaching critical brain stem structures may result in a circuitous sojourn through different anatomical pathways (represented by wavy arrows) with delay in development of clinical manifestations and long IP.

**Fig.4:**
A: Whole brain section of an adult mouse inoculated intracerebrally with CVS strain of rabies virus shows viral antigen deposition in cortex (C), hippocampus (H), thalamus (T), putamen (P), amygdala (A) and hypothalamus (Hy). [Internal capsule (IC)] Immunoperoxidase for rabies viral nucleocapsidx15
B: These areas correspond to areas rich in cholinergic innervation reflecting strong affinity when compared with section stained with choline esterase (B). Choline esterase histochemsitryx15

**Fig.5**
A: Pyramidal neurons of cerebral cortex show multiple characteristic eosinophilic intracytoplasmic Negri bodies (arrows). HEx240
B: Microglial nodules surrounding a degenerating neuron (arrow). Note absence of Negri bodies within the degenerating neuron.  

C: Dense perivascular lymphocytic cuffing around the parenchymal vessels spilling into the adjacent parenchyma of the brain stem in a case of rabies encephalitis.  

D: Rabies viral nucleocapsid antigen seen as fine, stippled deposits and occasional larger aggregates corresponding to Negri bodies within the cytoplasm within the magnocellular neurons of the brain stem reticular formation.  

E: CD68 positive macrophages around a degenerating neuron – neuronophagia.
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As expected for an atlas, the chapters are brief, typically 1–2 pages in length. The text is for the most part laudably concise, and highlights important features needed for making the diagnosis, without providing excessive detail. For those inclined to dig deeper, a good, basic bibliography is provided at the end of the book. Macroscopic images are provided for each entity, with gross, cytological and/or immunohistochemical images for many entities as well. As a new feature with the 2nd edition, all images may now also be accessed through an online searchable image bank.

While the overall utility and quality of the book is very good, there is some unevenness between sections, as is often the case in multi-authored works. Our favourites include the sections on infectious agents and paediatric lung disease, while others such as those on forensic pathology, pulmonary hypertension and therapeutic effects could benefit from significant revision in future editions. Likewise, there is some variability in image quality, with occasional images appearing to be of low resolution or slightly out of focus.

On the whole, this book has much to commend it to practising pathologists and trainees alike, as a useful quick reference guide and introduction to the field of pulmonary pathology that covers a very good breadth of both common and rare conditions. Those who already own a copy of the 1st edition may not find the extent of revisions sufficient to justify the cost of upgrading to the 2nd edition, but should keep in mind the updates noted in the references below.

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NOTICE
doi:10.1136/jcp.2006.045682.corr
Withdrawal of paper
The authors of the following paper “Tracking the footprints of the rabies virus: are we any closer to decoding this elusive virus?”, have withdrawn their paper following an allegation of plagiarism (J Clin Pathol on August 28, 2008; doi:10.1136/jcp.2006.045682.corr).