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THE PATHOGENESIS OF BITING ATTACKS OBSERVED IN EXPERIMENTALLY
INFECTED RABID SKUNKS DURING EARLY STAGE RABIES

A Thesis
Presented to
The Faculty of Graduate Studies
of
The University of Ottawa

by
Nonie L. Smart

In partial fulfilment of requirements
for the degree of
Doctor of Philosophy



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UNIVERSITÉ D'OTTAWA
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ABSTRACT

THE PATHOGENESIS OF BITING ATTACKS OBSERVED IN EXPERIMENTALLY INFECTED RABID SKUNKS DURING EARLY STAGE RABIES.

The proposal that the biting attacks which occur during furious rabies infection are due to selective infection of CNS neurons was studied in skunks (a species important in naturally occurring disease). In this model, skunk street rabies virus infection generally produces furious rabies (characterized by increased activity and biting attacks) while Challenge virus standard (CVS) infection results in dumb rabies (absence of biting and marked depression). A detailed immunohistochemical study of brains of skunks experimentally infected with either CVS or skunk street rabies virus revealed only trace amounts of viral antigen in limbic system neurons and marked differences in viral distribution between skunk street rabies and CVS rabies viruses. These data were collected during early stage rabies when behavioral changes occur. Areas which contained heavy accumulation of street virus but low amounts of CVS rabies virus were the neuronal perikarya and processes of the dorsal motor nucleus of the vagus, dorsal raphe nucleus of the midbrain, hypoglossal and red nuclei. In contrast, large accumulations of CVS rabies virus were found in the Purkinje cells of the cerebellum, the habenular nuclei, and in the pyramidal cells throughout the cerebral cortex, while corresponding areas in all street virus-infected skunks contained minimal antigen. These findings were very consistent for animals of the same

experimental group and between skunks inoculated both intramuscularly and intranasally with skunk street rabies virus. Skunks inoculated intramuscularly with CVS rabies virus failed to develop rabies. The correlation between virus infection of neurons and a possible pathogenesis of neural dysfunction was further studied using immunoperoxidase staining for the evaluation of CNS serotonin and met-enkephalin in skunks infected with either CVS rabies virus or skunk street rabies virus. There was no difference in the intensity of serotonin immunoperoxidase staining between uninfected control skunks or those infected with CVS or street rabies viruses. However, there was a reduction in the intensity of immuno-peroxidase staining for the neurotransmitter met-enkephalin, for rabid (both CVS and street rabies viruses) as compared to uninfected control skunks. These data showed that differences in the distribution of CVS rabies virus as compared to skunk street rabies virus in the CNS of experimentally infected rabid skunks, may account for the different clinical syndromes associated with these two viruses. The correlation of rabies virus accumulation within neurons to neurotransmitter imbalances during rabies disease requires further investigation to determine the mechanism by which this may occur.

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List of abbreviations

Ach - acetylcholine
AchR - acetylcholine receptor
A.D.R.I. - Animal Diseases Research Institute
BHK - baby hamster kidney
CNS - central nervous system
CVS - challenge virus standard rabies virus
DA - dopamine
DAB - 3,3'diaminobenzidine
EEG - electro-encephalogram
EMC - encephalomyocarditis virus
FBS - fetal bovine serum
G - glycoprotein (rabies virus)
GABA - gamma-aminobutyric acid
HEP - high egg passage
HSV - herpes simplex virus
L - RNA dependant polymerase (rabies virus)
M - matrix protein (rabies virus)
N - nucleoprotein (rabies virus)
NA - noradrenaline
NS - phosphoprotein (rabies virus)
REM - rapid eye movement
RER - rough endoplasmic reticulum
RNA - ribonucleic acid
RNP - ribonucleoprotein
TBS - tris buffered saline

Tris - tris automation buffer

5-HT - 5-hydroxytyptamine (serotonin)

CHAPTER 1

Introduction

General Introduction

Throughout the centuries, the hallmark of rabies has been the dramatic behavioral changes seen in animals during the clinical course of the disease. Historical records dating from ancient China and Mesopotamia in the 23rd century B.C., document "mad dog" disease as a fearsome and fatal condition (Wilkinson, 1988). Even the vilest of remedies was considered better than falling victim to rabies, such that treatments including hot oil baths, caustic wound treatments and the consumption of elixirs containing unusual plant and animal extracts found their place in the popular medicine of the century (Wilkinson, 1988). Despite the persistence of rabies in the population and the efforts of early investigators, it was not until the 1800's that scientific fact began to replace the folklore surrounding rabies. The experiments of Galtier in 1879 followed by those of Pasteur (which commenced in 1881), confirmed that rabies was caused by an infectious agent. In separate experiments, both of these investigators showed that rabbits could be infected experimentally with rabies by the injection of saliva taken from rabid dogs (Wilkinson, 1988). Shortly after this discovery Pasteur introduced the first rabies vaccine.

In the 100 years since Pasteur's experiments, techniques such as cell culture, electron microscopy and monoclonal antibodies have been used to characterize the structure and molecular biology of the virus. Yet, despite all of this accumulated information, we still have yet to determine how the virus causes the behavioral changes so typical of rabies. Hence the subject of this treatise; the study of the pathogenesis of behavioral changes in rabid animals, in particular, the biting attacks seen in rabid skunks.

Skunks appear to be well suited to this type of experiment because the clinical signs of aggressive behavior are much more distinct than in commonly used laboratory rodents, such as mice, and changes from normal behavior of the caged animals is more readily detected in skunks than in other wild species held in laboratory confinement. In addition, the skunk is an important vector of rabies in Canada and the United States and overall more diagnoses are made in this than in any other single species in these two countries (Charlton et al., 1988).

The study of the neural basis for aggressive behavior, in any animal model, is a difficult task that has challenged investigators for more than a century (Soubrie, 1986). The complex organization of the central nervous system, along with the myriad of activities and motivations underlying any behavioral repertoire, makes this type of study a most difficult and intricate undertaking. However, the substantial

impact of uncontrollable human violence on society (Valzelli, 1984) and domestic animal aggression on the economics and acceptability of modern animal agriculture (Fraser and Rushen, 1987), has continued to motivate researchers from many different disciplines to further investigate the subject of aggressive behavior. Experimental skunk rabies is a novel approach to the study of aggressive behavior, and, in addition to providing a means by which a selected aspect of rabies pathogenesis can be investigated, it may also provide some insight into aggressive behavior in other species.

Literature review. The pathogenesis of rabies virus in the central nervous system and perspectives on general mechanisms of aggressive behavior.

Molecular biology of rabies virus

Rabies virus is a member of the Family Rhabdoviridae. This Family which includes viruses that have a characteristic rod shaped configuration, is divided into 2 genera, Lyssavirus and Vesiculovirus (Baer et al., 1990). Rabies is the prototype of the genus Lyssavirus (meaning frenzy), while viruses classified as Vesiculoviruses (meaning vesicle forming) cause vesicular stomatitis. Often referred to as "bullet shaped", rabies viruses range from 130 to 300 microns in length and 75 microns in width (Tordo and Poch, 1988). The genome consists of a single helical strand of negative sense RNA which encodes the 5 genes; N, NS, M, G and L. The ribonucleoprotein (RNP) complex consists of viral RNA complexed with nucleoprotein (N), phosphoprotein (NS), and the RNA dependant polymerase L. These 3 proteins form the putative replication-transcription complex of rabies virus (Baer et al., 1990). Monoclonal antibody studies have shown that the amino acid sequence of N is highly conserved amongst rabies isolates (Wunner et al., 1988), and is considered to be the group specific antigen. Recently immunization of mice with the RNP complex has been shown to confer protection against lethal challenge by several Lyssaviruses (Dietzschold

et al., 1989). The matrix protein (M) and glycoprotein (G) are envelope-associated antigens. The M protein is located on the inner surface of the viral envelope, and is considered to play a role in the budding process since it is able to interact simultaneously with the developing RNP complexes and envelope lipids (Wunner et al., 1988). The transmembrane glycoprotein has been extensively studied since it was the first virus protein shown to induce protective virus neutralizing antibodies (Baer et al., 1990) and to function in the attachment of virus to the host cell (Tordo and Poch, 1988). Post-translational processing takes place in the endoplasmic reticulum, and the finished product consists of an inner tail, a transmembrane portion and spike like peplomers which protrude from the surface of the virus (Tordo and Poch, 1988). Two major antigenic sites as defined by monoclonal antibody studies, have been identified on the glycoprotein and these are called antigenic sites II and III (Lafon et al., 1991). In addition to these major antigenic sites, several other minor sites have recently been defined (Benmansour et al., 1991). Few mutations of antigenic site II have been shown to affect virus virulence but a mutation which replaces arginine 333 by any other amino acid besides lysine in site III may reduce or abolish pathogenicity for experimental infections of some but not all host species. (Tuffereau et al., 1989; Lafay et al., 1991). Other regions of the glycoprotein which determine virulence or virus neutralizing

ability have yet to be identified. Further study of the structure and function of the structural and nonstructural rabies virus proteins, will, unquestionably further our understanding of rabies virus pathogenesis. For a more detailed discussion of this subject, the reader is referred to the following review articles; Tordo and Poch, (1989), Wunner, (1988), Baer et al., (1990).

The basis for the differences in pathogenicity between street and fixed rabies viruses is still unknown. Virus which is obtained unpassaged from field cases of naturally occurring rabies, in all species, is referred to as street virus. Monoclonal antibody characterization of field isolates, has shown that street viruses form antigenically distinct groups which are often associated with a specific species and/or geographic location (Webster et al., 1986; Smith et al., 1986; Smith et al., 1988). Any street virus will become "fixed" after extensive passage through laboratory animals or cell culture. Louis Pasteur was the first to introduce this concept when he demonstrated that passage of street virus would shorten and "fix" incubation times as well as reduce the virulence of naturally occurring strains. Virus thus produced formed the basis for the first rabies vaccines (Bunn, 1988). Fixed viruses have also been used extensively in studies of experimental rabies since they are less pathogenic (reduce the risk to laboratory personnel) and fixed incubation results in more uniform experimental groups.

The first fixed strain was developed in Pasteur's laboratory and was named Challenge Virus Standard (CVS). It originated from a rabid cow and is still used today in studies of experimental rabies (Bunn, 1988), including the one reported here. Some other examples of fixed virus strains include the Fleury and Street Alabama Dufferin (SAD) strains. The Fleury strain was isolated from a girl who died of rabies in 1939 and was passaged 138 times in day-old chicks plus at least 205 times in embryonated eggs before vaccine preparation. The Street Alabama Dufferin strain originated from a rabid dog in 1935 and was passaged in mice. Many fixed strains of street viruses have been developed and several are still used in the preparation of present-day vaccines (Bunn, 1988). Vaccine-induced rabies has occurred when strains have not been sufficiently passaged to reduce their virulence (Bunn, 1988). Such miscalculations have occurred in part due to different species susceptibilities to the rabies virus.

It has not been determined why street virus infections are associated with increased virulence (Smith, 1982), longer incubation times (Smart and Charlton, 1992) and often a reduced inflammatory response in the CNS (Fekadu and Shaddock, 1984; Charlton, 1988) as compared to fixed virus infections. Clinical signs of rabies may also vary between street and fixed rabies virus infections in experimental animals and it has been proposed that these differences are strain associated

(Gourmelon et al., 1991; Smart and Charlton, 1992). Investigations which concern the genetic basis for differences in pathogenicity among rabies virus strains are still considered to be preliminary. Nucleotide sequence studies have revealed sequence differences between different rabies virus genomes, but the relevance of these findings to pathogenicity has not yet been determined (Tordo et al, 1988; Conzelmann et al., 1990).

Rabies disease

Rabies virus is found throughout the world and is known to infect all warm-blooded animals, although susceptibility to infection varies among species. Some animal vectors of enzootic wildlife rabies such as skunks or raccoons, are considered to be moderately susceptible to infection while others (foxes) are highly susceptible (Baer et al., 1990). Other factors which may influence the course of disease include; age and immunocompetence of the host (Lodmell and Ewalt, 1985), dose (Jackson and Reimer, 1989), route of inoculation (Fekadu et al., 1982; Jackson and Reimer, 1989), and strain of virus inoculated (Fekadu et al., 1982; Dietzschold et al., 1985; Tolson et al., 1990). Naturally occurring rabies is usually transmitted through bites by rabid animals. Rarely, the disease is transmitted by intranasal and corneal routes. Experimentally the disease can be transmitted by several routes including intramuscular, oral, respiratory,

intraperitoneal, intracerebral, subcutaneous (Charlton, 1988), and intraocular inoculation (Coulon et al., 1989). From an intramuscular site, virus may enter the axon immediately or after a period of replication at the site of inoculation (Baer, 1975; Charlton, 1988).

The neurotropic nature of the virus and its distribution within the nervous system has led to speculation that a neurotransmitter receptor may function in the attachment of rabies virus to the host cell (Gosztonyi, 1984). Heavy virus accumulations at neuromuscular junctions led to investigation of the acetylcholine receptor (AChR) as the host cell receptor for rabies virus (Lentz, 1982). Subsequent study revealed that alpha-bungarotoxin which also binds to the AChR, competitively reduced rabies binding at the neuromuscular junction (Lentz, 1982). Rabies virus glycoprotein was shown to have sequences homologous to the putative AChR binding site of snake neurotoxins (Lentz et al., 1984). Additionally, binding of rabies virus to purified Torpedo electric organ acetylcholine receptor was shown (Lentz, 1986). However, rabies virus can attach to a large number of different neuron and cell types (Kucera et al., 1985; Charlton, 1988), and this has led researchers to question whether the AChR is only one of many receptors or if it is a receptor for rabies virus at all. Reagan and Wunner (1985) stated that rabies virus-cell interactions were independent of the ACh receptor because anti-rabies antibodies did not bind to alpha-bungarotoxin and

the absence of Ach receptors did not impede the binding of rabies virus. Other suggestions for potential cell receptors include phospholipids, gangliosides and sialic acid residues (Tsiang, 1988).

The virus is carried towards the CNS by retrograde axonal flow, at a rate of approximately 75 mm per day in vivo (Dean et al., 1963) and 12 to 24 mm per day in vitro (Tsiang et al., 1979). Recently, Tsiang et al., (1989) demonstrated that anterograde transit also occurs at the rate of 100 to 400 mm per day in vitro. Substances which interfere with microtubule function such as colchicine, have been shown to block retrograde migration (Tsiang, 1979; Ceccaldi et al., 1987) but not replication of the virus (Conti et al., 1990). Since axons lack ribosomes, it is unlikely there is any viral replication during transit to the CNS. In the CNS, viral replication occurs in neurons and appears to spread via cell-to-cell transfer. Budding of virions on dendrites or neuronal perikarya with simultaneous uptake by axon terminals was demonstrated electron microscopically by Iwasaki and Clark, (1975) and Charlton and Casey (1979b). It has also been shown that in the early stages of intracerebral infection, virus spreads in a pattern consistent with the functional neuronal connections of the site of inoculation (Gillet et al., 1986; Jackson and Reimer, 1989). It is unlikely that the cerebrospinal fluid (CSF) serves a major role in the spread of virus within the CNS as virus is infrequently isolated from the CSF.

Simultaneous to CNS infection, virus spreads centrifugally via peripheral nerves to many body organs (Charlton, 1988; Schneider, 1991). Virus is often present in the salivary gland prior to clinical signs, and this plays a key role in the transmission of rabies virus to other susceptible hosts. (Fekadu, 1984; Charlton, 1988).

Early stage rabies is characterized by the onset of behavioral changes which are thought to occur as a result of viral replication in CNS neurons; however, the mechanism of these behavioral changes is not known. Rabid animals are classified as having either the furious or dumb form of rabies. In carnivores, in which classical rabies was probably first described (Wilkinson, 1988), furious rabies is characterized by hyperactivity, hyperirritability, and biting attacks directed towards other animals or objects within range, whereas this excitable and aggressive phase is absent in dumb rabies. Generally, the behavior change seen at the onset of dumb rabies is one where animals become more withdrawn or more sociable and affectionate (Fekadu, 1991; Bunn, 1991). Paralysis of the laryngeal muscles may be common to both forms and results in high pitched vocalizations and/or excessive drooling and dysphagia in all species (Fekadu, 1991; Bunn, 1991). In carnivores, third eyelid prolapse and a dropped jaw due to paralyzed masseter muscles are also common (Fekadu, 1991), and generally are accompanied by varying degrees of in-coordination, ataxia and progressive paresis.

In late stage rabies, where the animal is recumbent and often comatose and seizing, these clinical forms are indistinguishable. Death is commonly due to respiratory or cardiac arrest (Gourmelon, 1986). In experimental rabies, animals may die without any apparent clinical signs of rabies (Fekadu, 1984).

Skunk rabies is quite similar to that seen in carnivores. Charlton et al. (1991) described skunks with furious rabies as having increased alertness with activity levels ranging from restlessness to mania, exaggerated responses to stimuli, an apparent lack of fear and biting which is directed at any object or intruder within range. Some skunks showed muscle tremors, hypermetria, and in late stage rabies seizures, paddling and paresis followed signs of inco-ordination, weakness and ataxia. Skunks with the paralytic form of the disease were quiet and unresponsive in the early stages of the disease. Excessive drooling was an uncommon clinical sign.

In the past, studies of experimental rabies have most commonly utilized small laboratory animals such as rats and mice, yet the clinical signs of rabies in these species are not entirely consistent with rabies infection of common wildlife and domestic species. Rodent rabies is usually characterized by dumb rabies where lethargy, piloerection and progressive paralysis followed by coma and death, are the predominant clinical findings (Winkler, 1991). Furious rabies is uncommon in this species and unobserved in the laboratory

at the Animal Disease Research Institute (A.D.R.I.) in over 20 years of inoculations of rats and mice for rabies diagnostic and research purposes (at least 20,000 rodents per year) (Casey, 1991; personal communication).

Rabies in domestic animals in Canada, usually occurs as a result of infection by rabid skunks or foxes (Charlton, 1991). Although the clinical symptoms are similar to those already described, these animals may display species-associated clinical signs. Commonly noted signs of rabies in swine include, a sudden onset of inco-ordination, dullness, twitching of the nose, rapid chewing movements, excessive salivation and extreme pruritis of the hind quarters (Morehouse, 1981). Furious rabies is rarely observed in pigs. Furiously rabid cattle are seen to paw the earth, bellow continuously, and are extremely agitated, as compared to dumb rabies where the predominant signs are drooling, a vacant stare, anal straining with lack of anal tone, knuckling over of hind limbs, tail paralysis and genital excitement. Sheep and goats are similarly affected but may also exhibit signs of extreme puritis and sexual excitement (Timoney et al., 1988). Whether these differences in clinical expression of rabies between species is host- or virus-determined is unknown at this time.

The majority of cases of human rabies result from biting attacks by rabid animals (Flamand et al., 1987). The clinical progression of rabies in humans is similar to that seen in

animals. Both the furious and dumb forms of rabies occur and are sometimes referred to as encephalitic or paralytic, respectively. About 80% of patients develop furious rabies with the associated signs of hydrophobia, mental agitation, hallucinations, thrashing and other bizarre behavior which then deteriorates into stupor followed by coma (Baer, 1990). Patients with the paralytic form, succumb to progressive paralysis but retain their mental faculties and rarely experience hydrophobia. Other systemic disturbances may occur during the clinical course of the disease, such as autonomic, respiratory, and cardiovascular dysfunction; the reader is referred to Dupont and Earle (1965) and Baer (1990), for further details on human rabies.

The coincidence of early stage rabies with the aggressive behavioral changes of rabid animals facilitates the propagation of rabies because the opportunity for transmission of rabies virus during a biting episode is increased. High titres of virus are usually present in the saliva during this time (Charlton, 1987). Animals with dumb rabies, although unlikely to initiate a biting attack may be attacked by uninfected animals when they wander beyond their territory or fail to exhibit normal social behaviors. During the evolution of rabies virus, it is possible that the behavioral changes have evolved to facilitate the transmission of the virus via infected saliva. It is completely unknown as to how the rabies virus might interfere with neuronal cell metabolism

such that normally unaggressive animals display dramatic and uncontrollable biting attacks. Neither in vitro nor in vivo studies have determined whether this effect is specific to particular brain regions or if it results from a non-specific effect that involves the entire CNS.

Rabies virus and cell dysfunction

The enigma of rabies as well as many other CNS infections is the possible relationship of virus-induced neuronal injury to the clinical signs of disease. In vivo studies of the mechanism of neuronal cell dysfunction in viral encephalitides are relatively recent. This is probably due to a requirement for technology which is capable of measuring minute quantities of extremely labile substances that must be quickly extracted from the brain prior to post mortem autolysis. Lycke et al. (1970), measured the effects of several neurotrophic viruses on monoamine metabolism in the CNS. Mice were inoculated intracerebrally with either vaccinia, herpes simplex (HSV), pseudorabies, rabies, encephalomyocarditis (EMC), or influenza viruses and assayed for dopamine (DA), 5-hydroxytryptamine (5-HT), noradrenaline (NA), and their major metabolic end products in total brain homogenates. An increased release and synthesis of DA and 5HT was observed for all viruses. After further study of HSV, Lycke and co-workers (1972) concluded that virus infection stimulated increased neuronal activity and release of additional monoamines, since there was no evidence of impaired elimination or reduced uptake of amines. Unfortunately, these findings were not sufficient to explain the cause of the clinical signs seen in these encephalitic diseases. In the early stages of disease, neurotrophic viruses are often detected in specific regions rather than diffusely throughout the brain (Jackson and Reimer, 1989;

Johnson, 1982), thus whole brain extract measurements are not sensitive enough to detect regional neurotransmitter changes which might occur in areas of virus accumulation. For example in Borna disease, the destruction of specific groups of neurons accounts for the symptoms of this disease. Infection of tree shrews with this agent leads to a disturbance of the normal social behavior associated with female reproductive activities (Sprankel et al., 1978). The Borna disease virus preferentially infects limbic system neurons in this species and it was first believed that infected neurons were killed as a result of direct viral damage (Narayan et al., 1983). However, in 1989, Stitz and co-workers demonstrated that the cell destruction was, in fact, due to the host immune response to virus infection. They demonstrated that cyclophosphamide immunosuppressed animals failed to develop the pathological alterations of Borna disease. Disruption of normal cellular physiology may also occur in the absence of light or electron microscopic evidence of cell damage or death. In 1988, Lipkin et al., showed that acute Borna disease was associated with decreased levels of neural transcripts of cholecystokinin, somatostatin, and glutamic acid. These then increased back to control levels in recovered rats. There is a growing body of evidence that neurotropic viruses or agents can interfere with neurotransmitter metabolism in neurons of the CNS in the absence of histologic injury (Oldstone et al., 1977; Pocchiari et al., 1985; Lipkin et al., 1988).

This may be the case in rabies infected neurons since virus infected CNS neurons rarely show histologic evidence of cell damage; however, the exact nature of the functional alterations caused by rabies virus are still unknown. (Perl, 1991; Tsiang et al., 1991). Whether the virus disturbs cell function directly or as a result of the host immune response is still a matter of some controversy. The latter possibility will be further discussed in the following section. There have been several reports that suggest that rabies virus-induced interference with information transmission could account for the bizarre behavioral changes seen in rabid animals (Iwasaki et al., 1985; Gourmelon et al., 1986; Gourmelon et al., 1991). Changes occurring in neurotransmitter metabolism (synthesis, transport, release or degradation) or neurotransmitter receptor functions are potential mediators of this effect (Tsiang, 1988). Rabies virus infection does not appear to significantly depress host cell synthesis of RNA, DNA or proteins below pre-infection levels (Madore and England, 1977). However the effect of rabies virus on cellular luxury functions requires further investigation. In vitro studies have failed to elucidate a universally acceptable explanation for rabies-induced cell dysfunction. Some specific virus-induced effects have been identified in isolated experimental systems. Munzel and Koschel (1981) showed that persistently infected mouse neuroblastoma-rat glioma hybrid cells had a decreased affinity

for opiate agonists which was not accompanied by a decrease in the number of these receptors. These persistently infected cells were also shown to be unable to couple the opiate receptors to the inhibitory regulatory protein Ni of the adenylate cyclase (Koschel and Munzel, 1984). It was observed that receptor function as measured by c-AMP levels after prostaglandin stimulation was also impaired in mouse neuroblastoma-rat glioma cells which possessed receptors for catecholamines and acetylcholine (Ach). Thus Koschel and Munzel (1984) concluded that rabies virus interfered with the regulation of c-AMP via membrane receptors, by an unknown mechanism. The possible relationship of these findings to the clinical signs of rabies was not further explained. In a brief publication, Ceccaldi and co-workers (1990), demonstrated a reduction in the number of 5HT-1D serotonin receptors in the cortex and striatum of rabid rats. It was not stated in this abstract whether the infections were due to street rabies virus or CVS rabies virus. The binding of rabies virus to cellular receptors or interference with neurotransmitter metabolism are two mechanisms by which rabies virus could interrupt information transmission in the CNS. It has not been clearly shown if neural dysfunction in rabies involves either pathway.

In vivo studies are few and inconclusive, but data from these investigations also support the hypothesis that derangements in neurotransmitter physiology may account for

the clinical signs observed. Baride and Gaitonde (1980) inoculated mice intracerebrally with fixed rabies virus and measured the levels of NA, DA, and 5HT in whole brain extracts. Free amino acids and neurotransmitters were measured pre- and post-inoculation and at various times during clinical stages of disease. They found that free amino acids increased coincident with progression of the disease and NA and DA were depleted in the advanced stages of the disease. Unfortunately, due to poor experimental design and gross measurement of whole brain neurotransmitter levels, the data from this experiment are not likely to be valid.

In a unique in vivo study, Gourmelon et al. (1986), described the spontaneous changes in brain electrical activity associated with the spread of CVS rabies virus (fixed virus) in the CNS of experimentally inoculated mice. The initial phase was characterized by rapid eye movement (REM) sleep disappearance. At this stage, rabies virus was detected in the pons, cerebellum, thalamus and cerebral cortex. Clinically, mice were unstable, ataxic, apathetic and piloerected. In the second or mature phase, where mice were paralytic and recumbent, virus extended into the hippocampus and there was associated, generalized electro-encephalogram (EEG) slowing. The terminal stage ended with a flattening of cortical activity which ceased completely 30 minutes prior to cardiac arrest. The authors concluded from these data that, rabies is a disease of CNS functional alteration by a

mechanism that has yet to be discovered. Recently, Gourmelon et al., (1990) repeated the above experiment in mice infected with street virus. They found that in comparison to CVS infection EEG activity was preserved until the terminal stages of disease when it decreased suddenly and within a few hours became completely disorganized. Sleep and waking stages showed only minor changes throughout the course of disease. Cyclophosphamide immunosuppressed mice showed exactly the same pathophysiological changes. Unfortunately, the clinical features of the disease were not described for these mice. Although the immune component of the pathophysiology was addressed by immunosuppression of street virus infected mice, further investigation of the involvement of the immune system is necessary because the inflammatory reaction to rabies virus in the CNS depends on many factors (Iwasaki et al., 1977; Fekadu, 1982). For example, it has been proposed that the intensity of the cellular response in the CNS to rabies virus in humans determines whether encephalitic or paralytic rabies occurs (Hemachuda et al., 1988; Sriwanthana et al., 1989). Rabies in cows, on the other hand, is usually characterized by very little evidence of an inflammatory response in the CNS (Charlton, 1988). Thus immunosuppression experiments in some species may not reveal much about rabies pathogenesis if the level of CNS inflammation does not exceed a biologically significant threshold (which at this point is unknown).

The possibility that rabies virus might cause functional

alterations in non-neural areas of the CNS was investigated by Torres-Anjel (1986). He observed that animals inoculated with rabies virus showed a growth depression or wasting which could not be explained on the basis of reduced food intake. Immunoperoxidase was used to visualize "remarkably high levels" of virus in the pituitary hypophysis and serum levels of somatotrophin were reduced. The hypothesis that rabies virus induced wasting related to central hormonal dysfunction was poorly supported by the use of only 3 experimental animals and lack of negative controls in the immunoperoxidase staining procedures. Rabies virus has not been detected in the pituitary of experimentally infected rabid skunks (Smart and Charlton, 1992).

The pathogenesis of behavioral changes in rabid animals.

It is still a matter of some debate as to whether the dumb or furious form of rabies results from differential accumulation of virus in specific brain nuclei or virus dependent differences in the host immune response to rabies infection. The two theories are discussed below.

Virus distribution

In 1965, Johnson proposed that the selective vulnerability of neurons of the limbic system to rabies virus caused the bizarre behavioral activities seen in rabid animals. The limbic system contains many structures, some of which are implicated in emotionality and aggressive behavior as demonstrated in man (Valzelli, 1984; Soubrie, 1986) and many experimental animal models (De Lahunta, 1983; Pucilowski and Kostowski, 1983). In Johnson's (1965) experiments mice were inoculated with CVS virus and symptoms consistent with paralytic rabies were described. Virus was detected using immunofluorescence, a technique which is associated with rather poor anatomical detail. Thus, Johnson's claims require further clarification with respect to the exact location of virus accumulation in the limbic system and the pathology associated with the furious as well as the dumb form of rabies.

With the immunoperoxidase technique which was developed early in the 1980's, it is possible to visualize antigen

precisely within neurons and maintain anatomical reference in treated sections, due to formaldehyde fixation and counterstaining of the tissue (Hsu, 1981). This technique is valuable for the detection of rabies virus because brain regions such as midbrain or limbic system contain many small structures only some of which might contain virus and be involved in the pathogenesis of behavioral changes in rabid animals. Rabies virus has been detected in post mortem brain tissues using the immunoperoxidase technique with a primary antibody to RNP (Feiden 1985; Bourgon, 1987). Polyclonal or some monoclonal antibodies are able to bind with many strains of rabies virus (Feiden, 1985), probably due to the highly conserved sequence of the viral RNP antigen (Baer, 1990). Specimens from terminal cases of human rabies contained high levels of virus in diencephalon, brainstem and hippocampus, as well as virus in other areas of the brain (Feiden, 1985). Unfortunately, no clinical symptoms were included in this study and the description of virus location within the CNS was incomplete because only a limited number of brain sections were studied. The source of infection for these human cases was assumed to be bites from rabid dogs. In 1988, Feiden et al., summarized rabies virus distribution in the CNS from terminal wildlife cases submitted for diagnostic testing. The greatest accumulation of virus was found in the diencephalon, hippocampus and brainstem, however, rabies virus was diffusely present throughout the brain. Eight different wild and

domestic species were included in the summary of these data, thus species differences with respect to virus distribution could not be identified. Furthermore, there was no information on the behavioral activities of these rabid animals prior to their demise. In terminal rabies cases, the dumb and furious clinical forms are indistinguishable and the distribution of virus is very diffuse as compared to early stage rabies when clinical signs are first apparent (Jackson, 1991). Although there are some reports on the pathogenesis of street infection of laboratory rodents (Tsiang and Guillon, 1981; Lodmell and Ewalt, 1985; Gourmelon et al., 1991), there are no published studies on the distribution of street rabies virus in the early stages of naturally occurring infection. Thus, an appropriate model for the study of naturally occurring rabies would be one in which a vector species (supports enzootic rabies in nature) is infected with its species adapted street virus.

Fixed virus infections have been studied in greater detail. In 1989, Jackson and Reimer described a comprehensive study of the pathogenesis of CVS virus infection of mice. After mice were inoculated either intracerebrally or intrapedally, the location of virus was followed by immunoperoxidase staining at intervals of 24 hours post-inoculation. At the time of clinical signs, about 6 days post-infection, Purkinje cells and cerebral cortex contained marked amounts of antigen, whereas the hippocampus did not

become infected until day 8. On day 4 post-inoculation, at least 2 days prior to clinical signs, antigen could be detected in the CNS of footpad inoculated mice. The distribution of antigen at the onset of clinical signs was very similar between footpad and intracerebrally inoculated mice. These data do not substantiate Johnson's claim that bizarre behavior occurred as a result of selective infection of limbic system structures (1965). Many limbic structures such as the hippocampus were infected only late in the course of the disease.

In human rabies, the distribution of rabies virus and pattern of inflammation in the brains of terminal human rabies cases were considered to be unimportant in the clinical manifestation of the disease (Tirawatnpong et al., 1989). With the exception of the cerebellum, the amount of antigen in all brain regions studied was similar for both the encephalitic and paralytic forms of the disease. The pattern of inflammation did not parallel virus distribution in this study; however, the host immune response to rabies virus may be important in rabies pathogenesis.

Host immune response

It has been reported that the course of rabies disease may differ depending on the immunocompetence of the host (Kaplan et al., 1975; Lodmeli et al., 1985; Consales et al., 1990), as well as the type and intensity of cell mediated

immune response to the rabies virus (Iwasaki et al., 1977; Hemachuda et al., 1988; Sriwanthana et al., 1989). In addition, it has been shown that the same strain of rabies virus may lead to either dumb or furious rabies when inoculated into the same species (Fekadu and Shaddock, 1984). These observations support the notion that host factors may greatly influence the pathogenesis rabies disease.

Kaplan et al. (1975) showed that a potentially inapparent infection with a high egg passage strain of rabies virus (HEP) was lethal in athymic or cyclophosphamide immunosuppressed mice. Since cyclophosphamide inactivates both B and T cells, this experiment did not identify the exact components of the immune system that were the possible mediators of the effects observed. Iwasaki et al., (1977), showed that athymic mice developed antibodies in response to CVS rabies infection but cyclophosphamide treated mice were unable to mount a similar immune response to CVS infection. Immunocompetent mice had 20% less mortality but more severe paralysis and marked inflammation compared to immuno-compromised mice which had longer clinical courses and less inflammation in the CNS. These data suggest that encephalitic rabies is associated with a mild inflammatory response and paralytic or dumb rabies is due to the effects of a moderate to marked accumulation of inflammatory cells in the CNS. However, furious rabies has not been described in experimentally inoculated rabid mice, where the effects of immuno-suppression may be different.

Smith et al. (1982), also showed that cyclophosphamide immunosuppression of mice inoculated with street virus, increased the overall mortality rate and delayed onset of clinical signs and death for about 2 weeks. In mice, this street virus caused only paralytic signs. Passive transfer of homologous immune serum to immunosuppressed animals reversed this effect as shown by shortened clinical courses of disease.

Lodmell (1983), showed that the genetic makeup of mice determined their susceptibility to rabies infection. Backcross experiments showed that murine resistance is under the influence of two concurrently segregating genes (Lodmell and Chesebro, 1984). The basis for this resistance seemed to be a greater ability of resistant mice to restrict the multiplication of the virus in the CNS (Lodmell and Ewalt, 1985). Resistant mice also had higher antibody titers, and resistance was lost after treatment with cyclophosphamide. The macrophages of genetically resistant mice are slower antigen processors than the macrophages of susceptible mice. Therefore, it was proposed that macrophages which are slower to process antigen, present it more completely to the immune system, and thus these antibodies conferred greater protection against rabies (Consales et al., 1990).

The phenomenon of "early death", is also related to antibody production. It occurs in incompletely immunized individuals after infection with rabies virus; characteristically these animals die more quickly than non-

immunized infected controls (Blancou et al., 1980). It is believed to occur due to enhanced immunopathology in the CNS as a result of accelerated antibody production stimulated by immunization (Prabhakar, 1981). The mechanism by which antibody precipitates this phenomenon is not understood at this time but is apparently due to a B cell rather than a T cell effect.

Although the course of rabies has been shown to be affected by immunosuppression, it is as yet unknown if it is a determining factor in occurrence of either dumb or furious rabies. Charlton et al., (1984) immunosuppressed skunks with cyclophosphamide prior to inoculation with skunk street virus. Skunk street virus in skunks produces the furious form of the disease as characterized by biting attacks and aggressive behavior. There was no difference in the development of aggressive behavior in the immunocompromised versus immunocompetent skunks. The immunosuppressed animals had shorter incubation periods, rapid progression of clinical signs, zero or low levels of serum neutralizing antibodies, and few inflammatory cells in the CNS. The effect of immunosuppression on the length of incubation and progression of clinical signs in skunks infected with street virus, is opposite to some studies of CVS infection of immunosuppressed mice where incubation periods and progression of disease is lengthened (Iwasaki et al., 1977).

In a study of human rabies, Tirawatnpong et al., (1989)

suggested that virus localization may not account for the difference between encephalitic and paralytic rabies seen in humans. They proposed that the intensity of host T cell response determines the clinical outcome of rabies infection such that patients with high levels of CNS inflammation were more likely to experience the encephalitic form of the disease. Hemachuda et al., (1988), showed that patients with encephalitic rabies were more likely to have positive lymphocyte proliferation tests to selected antigens than paralytic patients who had no response at all. Sriwanthana et al., (1989) further examined lymphocyte subsets of human rabies patients and found that B cells were decreased in paralytic patients as compared to the encephalitic group. Rabies pathogenesis may differ between humans and animals because humans are almost always infected with street virus, whereas most experimental rabies infections in animals are due to CVS or fixed rabies virus. In addition, humans are always infected with a non-host adapted strain of street virus whereas dogs, foxes or skunks are most often infected with a species-adapted strain of street virus during naturally occurring rabies. It might be suggested that some of the controversy over the pathogenesis of behavioral changes in rabies infection is due to the likelihood that no single model of rabies infection is appropriate for all species.

Approaches to the study of aggressive behavior

The study of the neural basis for aggressive behavior is a difficult task that has challenged investigators for more than a century (Soubrie, 1986). This is largely due to several basic problems that are inherent in the design and interpretation of behavioral studies. It is difficult if not impossible to house laboratory animals under conditions similar to those which occur naturally. Unfortunately, even subtle environmental changes, which arise as a result of confinement conditions can lead to dramatic changes in the social behavior of experimental animals. This issue in particular, makes the interpretation of data problematic because it may be difficult to determine if the results are comparable to the activities of free ranging animals. A great deal of controversy in the published literature can be attributed to this fact alone. For example, the four most common laboratory models for studying biting are; predatory (rat presented with a dead or anesthetized mouse), shock induced (response to inescapable shock treatments), isolation induced (failure to learn normal social repertoire) and irritable (irritant induced) (Bell and Hepper, 1987). In addition, animals are often preconditioned (brain lesioned, trained to respond to given stimuli) prior to being tested in these artificial situations for aggressive response. Thus, in addition to inadequate paradigms, the pitfalls of current behavior research have been summarized as; narrow range of

species studied, inappropriate animal housing, inappropriate stimuli and lack of adequate detail in the recording and analysis of the behavior under study (Huntingford, 1980).

The present study is the first in which the skunk experimental rabies model has been used to study the biting aspect of aggressive behavior. It offers several advantages over traditional methods for investigating aggressive behavior because no invasive techniques such as brain lesioning are used and no modification of the environment (isolation rearing, shock treatments) are required to facilitate a biting response. In addition, the laboratory behavior of skunks when housed singly under laboratory conditions has been well documented in our facility and rabid skunks are clearly distinguishable from normal skunks either by their attacks on any object placed within their range or their dull and unresponsive attitude (Smart and Charlton, 1992).

Despite the apparent similarities in aggressive behavior between most species, the concept of aggressive behavior is not universally applicable between animal species (Avis, 1974; Moyer, 1968; Flynn, 1976). Aggressive behavior may arise in a variety of situations and consist many different components (biting, kicking, snarling), not all of which occur in all species (dogs don't kick, horses don't growl). Furthermore, it is likely that the neuronal circuitry differs depending on the "type" of aggression that is occurring (Bandler, 1988; Seigel and Brutus 1990). For this reason, it

is necessary to define aggressive behavior with respect to the paradigm under study (Avis, 1974; Flynn, 1976). In 1968, Moyer classified aggressive behavior into the following eight categories; intermale, fear-induced, irritable, predatory, maternal, sex-related, territorial and instrumental based on the motivation of the animal to perform this activity. Other similar systems have been developed, as reviewed by Moyer, (1968), but none are universally acceptable because of differences in animal species and the margin of interpretation that is required for grouping various activities into a motivational classification. It would be difficult if not impossible to classify the aggressive behavior seen in rabies, on a motivational basis, due to our incomplete understanding of the biology of this disease at the present time. For example, biting attacks could be due to an enhanced perception of threat or fear, increased territoriality, or some other cause which is completely unknown.

Instead of using a motivation as an approach to the study of aggressive behavior, more recent studies have focused on the neuronal circuitry associated with specific components (or combinations) of aggressive behavior such as biting, sympathetic stimulation or vocalization. This eliminates the need to classify the type of aggression occurring because only a small component of the aggressive act is being measured. Often the variable chosen for study (ie. piloerection or pupillary dilation) is much easier to identify and quantify

than an "aggressive response". Biting behavior of experimentally infected rabid skunks is easily detected in rabid skunks and is a consistent indicator of early stage rabies (Smart and Charlton, 1992). For this reason, the remaining discussion will be limited to biting associated with aggressive behavior.

Neuroanatomical correlates of aggressive behavior

It is generally accepted that biting elicited by the stimulation of CNS structures (in all species studied), occurs via two main pathways as summarized by Siegel and Brutus (1990). Quiet biting attack, which is predatory in nature, and often used to kill animals, is stimulated from a region which includes the rostral aspect of the lateral and preforical hypothalamus and extends posteriorly to the ventral tegmentum and ventral aspect of the midbrain periaqueductal grey and as far caudally as the lateral tegmental fields of the pons. Affective defense biting, on the other hand, is associated with pupillary dilation, vocalization, piloerection and other sympathetic systemic signs and is seen as a directed biting for defending territory or offspring, occurring in response to threat. It is elicited from stimulation of sites located throughout the rostrocaudal extent of the medial preopticohypothalamus and the dorsal aspect of the midbrain periaqueductal grey.

Facilitation of biting behavior may also occur due to the

ablation of CNS structures known to inhibit aggressive behavior. For example, lesioning of the midbrain raphe nuclei has led to increased muricide by rats (Yamamoto and Ueki, 1977; Pucilowski and Kostowski, 1983), presumably due to the release of a normally repressed response. The midbrain raphe nuclei are part of a group of brain structures commonly referred to as the limbic system, which collectively have a major function in modulating the emotional state of an individual (Angevine and Cotman, 1981; DeLahunta, 1983). The exact limits of the limbic system are a matter of some debate.

In 1937, James Papez proposed that the rhinencephalon had additional functions beyond its description as an olfactory centre (DeLahunta, 1979). In 1955, MacLean introduced the term "limbic system" and included the following phylogenetically ancient structures, olfactory cortex, hippocampus, cingulate and subcallosal gyri, amygdala, septum, hypothalamus, anterior thalamic nuclei and basal ganglia. Since that time, many other structures previously not considered to be part of the limbic system have been shown to be functionally interconnected with the limbic system. Angevine and Cotman (1980) also included septum, tegmentum and midbrain raphe nuclei and habenula to the list of structures. For this project, we have adopted the limits of the limbic system as described by Angevine and Cotman (1980).

Neurotransmitters associated with aggressive behavior

Many different methods have been used to study the role of neurotransmitters in aggressive behavior in vivo, either in whole brain or defined brain regions. These include; correlation of neurotransmitter levels with behavior, administration of an agonist to enhance neurotransmitter effect with measurement of levels of aggressive behavior, decreasing neurotransmitter action by the administration of various substances (antagonist, receptor blocker, synthesis inhibitor, storage depleter or neurotoxin). Manipulations within the CNS may affect more than one neurotransmitter system (Stewart 1989; Mitchell et al., 1990), but all of these changes have not yet been completely characterized.

Although rabies induced aggression may be due to many disturbances in the catabolism and/or metabolism of inhibitory neurotransmitter(s) or respective receptor malfunction, this study focuses on a putative reduction in detectable levels of selected inhibitory neurotransmitters as a mechanism whereby aggression is facilitated in rabid animals. In this context, two neurotransmitters (serotonin and met-enkephalin) were selected for study due to their known inhibitory effect in the CNS and their localization to the limbic system.

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine produced mainly by the raphe neurons of the midbrain, pons and

medulla. Other serotonergic cell bodies have been identified outside this region but their input to the CNS is minor by comparison (Vandermaelen, 1985). In 1983, Steinbusch delineated nine raphe serotonergic regions in the rat brain and designated them as B1 - B9. In general, groups B1 - B3 project caudally within the brainstem to the medulla and spinal cord while groups B4 - B9 project rostrally from the pons and midbrain. B7 (dorsal raphe nucleus) and B8 (median raphe nucleus of the midbrain) provide the major serotonergic input to the limbic structures of the diencephalon and telencephalon, such as the thalamus, hypothalamus, habenula, cerebral cortex, caudate nucleus, putamen, pallidum amygdala and septal nuclei. It is likely that the raphe neurons produce several neurotransmitters and recently it has been shown that many serotonergic cell bodies also produce met-enkephalin (Hunt and Lovik, 1982; Leger et al., 1986).

The dorsal raphe nucleus (the largest of all the raphe nuclei), is found in the ventral periaqueductal grey matter and raphe region of the midbrain. Ventrally it is located between the medial longitudinal fasciculi and extends caudally into the periventricular grey matter of the rostral pons and rostrally to the Edinger-Westphal nucleus located approximately at the level of the oculomotor nucleus (Tork, 1990). The exact margins of this nucleus and the other eight raphe nuclei show some variation between species. Pucilowski and Kostowski, (1983) demonstrated that of the two main serotonin

nuclei (dorsal raphe nucleus and median raphe nucleus), only the dorsal raphe nucleus was involved in the inhibition of aggressive behavior in their behavioral trials. These data support the notion that in spite of producing the same neurotransmitter, the role of each raphe nucleus on behavior may be variable.

One of the defining characteristics of central serotonergic neurons is their slow and steady rate of discharge which is directly proportional to the level of arousal of the animal. Thus serotonergic discharge reaches a plateau during wakefulness and almost completely disappears during REM sleep (Rogawski and Barker, 1985). Generally, serotonergic neurons are believed to play a key role in the inhibition of emotionally based behavior (Soubrie, 1986); however, different paradigms have produced conflicting results, which may be partially explained by the discovery of several different types of serotonergic receptors (Palacios, et al., 1984). These receptors have been classified into three groups, 5-HT 1, 2 and 3, which have been further subdivided; however, the classification is by no means complete. The limbic system contains many (but not exclusively) 5-HT 1(A-D) receptors, while 5-HT 2 are found in cerebral cortex and 5-HT 3 are excitatory receptors associated with the peripheral nervous system. The functions of these sites are still not completely understood. Some indication of function has been derived by binding of agonists and

antagonists and observation of behavioral modification (Glennon, 1990). Activation of the 5-HT receptor prevents neurotransmitter release from functionally connected neurons and therefore neuronal function is inhibited. Serotonin is removed from the synaptic cleft by an uptake mechanism. Inhibition of serotonin uptake results in enhanced serotonergic nerve transmission due to the excess of serotonin which remains in the synaptic cleft. This is also accompanied by a subsequent decrease in the production and release of serotonin, so that steady state levels are maintained in serotonin producing neurons (Tork, 1990).

Serotonin is derived from dietary L-tryptophan which is actively transported across the blood brain barrier as well as neuronal membranes (Bradford, 1986). The biosynthesis of serotonin is accomplished by the addition of a hydroxyl group to the 5 position on the indole ring (tyrosine hydroxylase) followed by decarboxylation (5-hydroxytryptophan decarboxylase). Serotonin is stored in neurons and axons in the form of granular vesicles. In addition to its association with emotive states, a reduction of CNS serotonin levels has been implicated as part of the patho-physiology of dementia associated with the following conditions; Alzheimer's disease, Parkinson's disease and Huntington's chorea (Cross, 1984).

Met-enkephalin

Met-enkephalin was discovered in the mid 1970's and has

since been characterized as an opioid peptide (Dingledine, 1985). It is derived from the precursor protein pre-proenkephalin. The pre sequence is directed into the rough endoplasmic reticulum (RER) and then the cleaved pro-protein moves to the cisterna of the RER. In the Golgi apparatus it is packaged into secretory granules, where final processing may occur at any time, including in axon terminals within synaptosomes (Stewart, 1989). For this reason, met-enkephalin immunoreactivity is most often detected in fibres and terminals (Haber and Elde, 1982). The final structure (Try-Gly-Gly-Phe-Met OH), is contained in many opioid peptides and this accounts for the cross reactivity seen between related opioid substances. Met-enkephalin is widely distributed in the CNS and considered to be an inhibitory neurotransmitter (Bradford, 1986). It inhibits neuronal firing, and it is proposed that it targets GABA neurons (Frederickson, 1982) as well as other neuroexcitatory substances (Kuznetsov and Godukhin, 1985; Bishop, 1991).

The highest concentration of met-enkephalin is found in the fibres and terminals within the globus pallidus and caudate nucleus, with moderate amounts in the amygdala, septum, hypothalamus and midbrain (interpeduncular nucleus and periaqueductal grey matter) (Frederickson and Geary, 1982). Species differences between rat and monkey have been reported (Sar et al., 1978). Met-enkephalin is proposed to be a neurotransmitter associated with nociception, mood regulation

and control of respiration. The regional distribution of this substance is consistent with these proposed functions (Haber and Elde, 1982).

It is generally accepted that injury to the CNS may alter the distribution and intensity of met-enkephalin staining; for example, after hippocampal kindling a marked increase in the met-enkephalin immunoreactivity of hippocampal mossy fibre terminals was observed (Wanscher et al., 1990) as well as in the rat superior colliculus after eye enucleation (Okamoto et al., 1990). The mechanism for the change in the distribution of met-enkephalin immunoreactivity after brain injury has not been determined. Met-enkephalin has also been shown to interact with many neurotransmitters such as dopamine, serotonin and gamma-aminobutyric acid (GABA) (Yang, 1984), and has been co-localized with serotonin, GABA as well as other neurotransmitters (Stewart, 1989).

Statement of purpose

This research project was initiated in order to further our understanding of the pathogenesis of aggressive behavior in rabid animals. The mechanism(s) by which this occurs is/are completely unknown. The purpose of the experiments which comprise this thesis, was to investigate the pathogenesis of biting attacks in rabid skunks. Previous work in our laboratory has shown that skunks experimentally infected with skunk street virus generally show the furious form of rabies while those infected with Challenge Standard Virus (CVS) or fixed rabies virus develop dumb rabies. Although the scientific basis for this is unknown, we propose that differences in the accumulation of street versus fixed rabies virus in neurons of the central nervous system (CNS) may account for the different clinical syndromes associated with these respective infections. Since one of the major differences between furious and dumb rabies is the biting attacks associated with furious rabies, brain nuclei which are involved in the pathogenesis of this behavior may contain greater quantities of street rabies virus and therefore might be more likely to dysfunction. A disinhibition of normally suppressed aggressive impulses could lead to the expression of biting attacks as seen in furiously rabid skunks.

These experiments were designed to answer the following questions.

1. What is the distribution of street rabies virus in the CNS of experimentally infected skunks during the early stages of rabies?

2 a) What are the differences in the distribution of skunk street rabies virus versus CVS rabies virus in the CNS of experimentally infected skunks during early stage rabies?

b) Are there any heavy accumulations of either strain of rabies virus in regions known to mediate aggressive behavior?

3. Does skunk street rabies virus or CVS rabies virus infection in vivo reduce the intensity of immunoperoxidase staining for serotonin and/or met-enkephalin in the central nervous system of experimentally inoculated rabid skunks?

CHAPTER 2

Materials and Methods

Experimental animals

The experimental animal for all trials was the striped skunk (Mephitis mephitis). All experimental groups contained animals of approximately one year of age with equal numbers of males and females. They were obtained from Ruby's fur farm in Iowa, and were of mixed genetic background. Prior to arrival at our laboratory they were descented. Upon arrival at the the Animal Diseases Research Institute (A.D.R.I.), they were housed singly in stainless steel cages which measured 40 X 53 X 62 cm. Cage banks were 3 tiers high and contained a total of 6 cages. In addition, skunks were vaccinated against distemper before arrival and treated for intestinal parasites at regular intervals during their stay in the isolation facility at A.D.R.I. All animals included in these studies were seronegative for rabies prior to the commencement of experimental procedures.

In Verts (1967), the normal behavior of the striped skunk held in captivity is described as follows. Skunks maintained singly in cages are usually placid but may run rapidly around the cage if intruded upon. The vocal repertoire includes a variety of hisses, growls, squeals, churls and cooes. Warning or defensive behaviors are exhibited when a skunk is cornered or surprised. A skunk will usually face an intruder with the

tail elevated and back arched and then stamp its front feet in unison. Sometimes this is accompanied by a lunge towards the intruder followed by a retreat and dragging of the front feet. This may be repeated several times in succession. If this fails to dissuade the intruder, the skunk will face away from the intruder and elevate its tail as if to spray. This posture is adopted even in descented, captive skunks.

All care and handling of skunks in these experiments followed the guidelines of the "Guide to the Care and Use of Experimental Animals", Volumes 1 and 2, of the Canadian Council for Animal Care. Particular attention was paid to ensuring that skunks were not subjected to unnecessary pain or discomfort during inoculation procedures and the clinical course of rabies. Skunks were anesthetized for all inoculation procedures by an intramuscular administration of 2 mg acepromazine maleate (Ayerst Laboratories, Montreal, P.Q.), 160 mg ketamine hydrochloride (Rogar STB Inc., London, Ont.) and 5 mg xylazine (Haver Lockhart, Etobicoke, Ontario). Except where animals died unexpectedly, they were humanely killed during the initial stages of rabies as described later in this chapter.

Virus preparation

The virus inocula were prepared in the following manner. For skunk street virus, a 10% w/v suspension was prepared from submandibular salivary glands taken from skunks that had died

from naturally occurring rabies that had been submitted to A.D.R.I. for rabies diagnosis. Gland suspensions were titrated for rabies antibodies and those with little or no titres were pooled together prior to further use. The diluent consisted of 0.1M phosphate buffer (pH 6.8) containing 10% fetal bovine serum (FBS), 1000 IU/ml of penicillin G potassium and 0.26 mg/ml of streptomycin sulfate. This was centrifuged at 2000 Xg for 20 minutes and the supernatant was saved and stored at -70 °C until use.

The CVS rabies virus inoculum was prepared by passing a stock culture of CVS-56 rabies virus containing 10^6 median tissue culture infective dose (TCID₅₀)/ml 3 times in BHK/C13 cells. The supernatant was harvested and centrifuged at 2000 Xg for 5 minutes and stored at -70 C until further use.

Viruses were titrated (dilutions 10^{-1} to 10^{-8}) immediately post inoculation in both tissue culture and by intracerebral inoculation of weanling Swiss white mice. Street virus was grown in neuroblastoma cells and CVS virus on BHK-21 cells, cell lines to which these viruses are particularly well adapted (Webster et al., 1988). The titre endpoint was considered to be the dilution at which 50% mortality was observed.

All viruses and biological materials were handled in accordance to the Medical Research Council Laboratory Biosafety Guidelines (1991). In Schedule VII of these guidelines, street virus is classified as risk group III,

while CVS is found in risk group II.

Inoculation procedures

All procedures used for inoculation were developed at A.D.R.I. by Dr. K. Charlton and his co-workers, and are routinely used at this institution for studies on experimental rabies (Charlton and Casey, 1979).

Intramuscular inoculation

The right hind foot of each animal was surgically prepared and the abductor digiti quinti muscle exposed. This muscle was inoculated with 0.3 ml of the 10% salivary gland suspension (street rabies virus) or CVS rabies virus as indicated. Negative controls received diluent only.

Intranasal inoculation

Skunks received 0.5 ml of street rabies virus or CVS rabies virus which was deposited by drops into the left nostril. Negative control skunks received diluent only.

Clinical protocol

Post inoculation, skunks were returned to their cages and observed twice daily for clinical signs of rabies. Specifically, demeanor (alert or dull), posture (normal or recumbent), gait (paralysis, ataxia or weakness) and aggressive behavior (approaches intruder, bites foreign

objects), were graded as 0, no change from normal behavior; +, mild; ++, moderate; +++, marked display of behavior in question. Skunks were considered to be showing early clinical signs of rabies when they showed signs of ataxia and incoordination and/or they would immediately attack and bite a wooden applicator stick presented to them through a small opening in the cage wall. Responses to this "stick test" were subjectively graded as: 0 (no attempt to approach or bite stick), + (mild interest and tentative chewing), ++ (rapid attack and severe biting), +++ (rapid attack and manic chewing until destruction of the stick). Skunks were humanely killed approximately 24 to 48 hours after the first observation of a positive response to the stick test and/or gait/postural changes such as ataxia or paresis. Skunks were anesthetized as described above and then killed by an intracardiac injection of phenobarbital or perfused with fixative. The carcass and internal organs of each skunk were grossly examined and the brain removed. In non-perfused skunks a small piece of brainstem was submitted to our diagnostic laboratory for fluorescent antibody confirmation of rabies. Control skunks and those which did not succumb to rabies after experimental infection, were humanely killed 45 days post infection. Each carcass was examined grossly and a piece of brain stem was submitted to our diagnostic laboratory for rabies diagnosis.

Fixation and tissue processing

Immersion fixation

For rabies virus detection, the whole brain was immersion fixed overnight in 0.1M phosphate buffered formalin pH 5.3 at room temperature. It was then blocked into transverse sections and processed into paraffin blocks using routine histological procedures.

Perfusion fixation

Perfusion fixation was required for the detection of serotonin and met-enkephalin by immunoperoxidase staining. Prior to this procedure, the anesthetized skunks were supplemented with the inhalent anesthetic AErrane (Anaquest, Mississauga, Ontario). When skunks were judged to be in a deep surgical plane of anesthesia, the thoracic cavity was opened and the heart exposed by removal of the pericardial sac. The left ventricular chamber of the heart was cannulated with a blunt ended 2 inch cannula and the right atria opened. A pre-rinse with 200 ml cold lactated Ringer's solution preceeded the flow of 2 l of perfusant at 4 °C. Skunks were perfused with 3.7% paraformaldehyde pH 7.3 by gravity flow for approximately 20 min. The whole brain was then removed and immersion fixed in the perfusion fluid, overnight at 4 °C. Tissues were then blocked and processed into paraffin embedded blocks using routine histological procedures.

For all fixation methods, the brain was serially

sectioned from caudal medulla to the rostral margins of the olfactory lobes of the cortex. At 100 micron intervals, two 5 micron adjacent sections were saved for staining. One section was stained with cresyl violet acetate (Aldrich, Milwaukee, Wisconsin) (Kluver and Barrera, 1953), while the other tissue was mounted on a gelatin-coated glass slide and subjected to the ABC immunoperoxidase technique.

Automation of the ABC immunoperoxidase procedure

The Fischer Histomatic Slide Stainer, Codon-Series (Lexington, Massachusetts) consisted of computer software, a robotic arm, reagent chambers, an oven and an incubation chamber. Custom manufactured slides were paired with tissue sections facing. A 75 micron space between slides facilitated the filling and emptying of this gap by capillary action. Solutions were drained when the fluid interface at the bottom of the slides was blotted onto an absorbent material. Using the components of this system, 60 slides (30 pairs) could be stained in approximately 3 hours. Special attention to the quality of the slides (ability to hold fluid) and to the temperature of the heating chambers was required to attain optimum staining results.

Detection of rabies virus by ABC immunoperoxidase staining

This method is based on a technique previously described by Hsu (1979). Antigen is bound by antibody labelled with a

peroxidase marker. When this marker is developed, the antigen/antibody complex is visualized as a brown/black substance. Paraffin embedded sections were dewaxed by incubating sections in xylene at 60 °C for 8 minutes, followed by 3 xylene washes, 3 alcohol washes, and 3 washes in Tris Automation buffer pH 7.4 (Tris), (Biomed, Foster City, California), all at room temperature. All incubations thereafter were for 30 minutes at 45 °C and were followed by 3 washes in Tris, unless otherwise stated. The staining procedure commenced with three, 0.1% pepsin digestions of 5 minutes each. Following digestion, the sections were then incubated in normal goat serum (Vector Laboratories, Burlingame, California), to reduce background due to non-specific staining. All reagents obtained from Vector Laboratories were used according to the manufacturer's instructions. This was followed by incubation with the primary antibody (diluted 1:1000) for 40 minutes at 45 °C. This antiserum was prepared in our laboratory by the inoculation of rabbits with purified CVS rabies virus RNP. The tissue sections were then incubated with a biotinylated linker antibody, (anti-rabbit IgG, raised in goat) (Vector Laboratories). Endogenous peroxidase was quenched by exposing the slides to a 1:20 hydrogen peroxide:methanol solution for 20 minutes at room temperature. Finally, the tissue sections were incubated with the avidin biotin complex (Vector Laboratories). The peroxidase was visualized by the

application of 0.2 mg/ml 3,3'diaminobenzidine (DAB), (Sigma, St. Louis, Mo.), containing 0.01% hydrogen peroxide for 4 minutes at room temperature. Sections were then counterstained in haematoxylin for 4 minutes. A known positive and a negative control slide were included in each staining run.

Immunoperoxidase stained sections were subjectively and blindly evaluated every 100 microns throughout the entire brain for the location and density of staining for rabies virus. Various nuclei/regions were graded according to the following scale: 0, no detectable antigen; +, antigen present in small amounts; ++, moderate antigen content; +++, marked antigen content; +++++, dense and heavy antigen content. A total of 50 regions or nuclei, as listed in Table 1, were compared between experimental skunks within and between trial groups. The adjacent sections stained with cresyl violet provided reference for anatomical location/identification.

Detection of neurotransmitters

Slides were dewaxed as described above. The slides were then rinsed in 0.05M tris buffered saline (TBS), which was used for all washes in this procedure. They were incubated with 0.4% Triton X-100 (Sigma, St. Louis, Missouri) at room temperature for 30 minutes. All incubations were carried out at room temperature, and were followed by three sequential 5 minute washes in TBS plus Triton X, unless otherwise stated.

Sections were blocked with 10% skim milk powder containing 0.1% Triton X for 1 h. Primary antibody obtained from Incstar (Stillwater, Minnesota) was used at a dilution of either 1:1000 (serotonin) or 1:150 (met-enkephalin) in 5% milk powder and 0.1% Triton X. Incubation was at 4 °C, in a moist chamber for 48 hours. After incubation was completed, the slides were warmed to room temperature before commencing with the next step. Biotinylated antibody (Vector Laboratories) was diluted in TBS with 5% milk powder and 0.1% Triton X (according to the manufacturer's instructions) was applied and the slides were incubated for one hour. In the final incubation, the tissue sections were incubated with the avidin-biotin complex (Vector Laboratories) diluted in TBS with 5% milk powder. The chromagen was developed with DAB and hematoxylin counterstain applied as for rabies virus. Slides were evaluated every 100 microns for the location and density of immunoperoxidase staining.

Table 1. Structures and nuclei of the skunk CNS included in the evaluation of location and intensity of immunoperoxidase staining for rabies virus antigen.

Gracile nucleus
Cuneate nucleus
Inferior olivary nucleus
Accessory cuneate nucleus
Spinal nucleus of the trigeminal
Dorsal motor nucleus of the vagus
Hypoglossal nucleus
Abducens nucleus
Cochlear nucleus
Facial nucleus
Motor nucleus of the trigeminal
Vestibular nuclei
Trochlear nucleus
Superior olivary nucleus
Nuclei pontis
Purkinje cells
Granular layer, cerebellum
Fastigial nucleus
Nucleus interpositus
Dentate nucleus
Red nucleus
Substantia nigra
Medullary raphe nuclei
Pontine raphe nuclei
Midbrain raphe nuclei
Dorsal raphe nucleus^a
Medulla reticular nuclei
Pontine reticular nuclei
Mesencephalic n. of the
trigeminal
Superior colliculus
Inferior colliculus
Interpeduncular nucleus
Ventral periaqueductal grey matter
Dorsal periaqueductal grey matter
Oculomotor nuclei
Pulvinar
Pineal body
Habenular nuclei
Hippocampal formation
Dentate gyrus
Thalamus
Nucleus subthalamicus
Hypothalamus
Mamillary bodies

Table 1 (continued)

Pyriiform lobe of cortex
Amygdaloid nucleus
Caudate nucleus
Septal nuclei
Cortex except pyriiform lobe
Olfactory lobe

a) Rostral margins, at the level of the oculomotor nuclei.

Experimental outline

In the first experiment of this study, skunks were intramuscularly inoculated with skunk street rabies virus ($10^{7.5}$ TCID₅₀/ml), observed for clinical signs of rabies and humanely killed when they were judged to be in the early stages of rabies. The brains were removed and immersion fixed in formaldehyde pH 5.3. The location and density of rabies virus antigen in the brains (Table 1) of these experimentally inoculated skunks was visualized by immunoperoxidase staining of paraffin embedded tissues. In the second experiment (comparison of the distribution of skunk street rabies virus with CVS rabies virus), it was necessary to repeat the inoculations using the intranasal route. Challenge Virus Standard rabies virus given intramuscularly in skunks has a very low infectivity rate. Skunks were inoculated intranasally with either skunk street rabies virus ($10^{8.5}$ TCID₅₀/ml) or CVS rabies virus ($10^{8.5}$ TCID₅₀/ml), observed for clinical signs of rabies and humanely killed when judged to be in the early stages of rabies. The skunks and their tissues were processed in exactly the same way as for the first experiment. The location and density of rabies virus antigen in the CNS was compared between these two rabies virus strains.

A third experiment was done in order to investigate a possible explanation for the CNS dysfunction of rabid animals. The intensity and distribution of immunoperoxidase staining of

either serotonin or met-enkephalin in the CNS of rabid skunks was compared to uninfected control skunks. Skunks were intranasally inoculated with either skunk street rabies virus or CVS rabies virus, observed for clinical signs of rabies and perfused with paraformaldehyde by intracardiac cannulation when they were considered to be in the early stages of rabies. The location and density of rabies virus antigen was determined as for the first two experiments and in addition, 2 adjacent sections were subjected to immunoperoxidase staining for either serotonin or met-enkephalin throughout the brain.

CHAPTER 3

The distribution of skunk street virus in the CNS of experimentally infected rabid skunks.

Introduction

The behavioral changes of rabid animals have been of interest to scientists for centuries. Ancient documents describe "mad dog" disease in which dogs suddenly became wild and vicious and were seen to foam at the mouth (Wilkinson, 1988). Although the furious form is by far the most dramatic clinical presentation of rabies, the dumb form of the disease also occurs. Despite the long history of rabies in man and animals, the pathogenesis of these behavioral changes are absolutely unknown.

In skunks, furious rabies is characterized by hyperactivity occasionally bordering on mania and biting attacks, as compared to dumb skunk rabies where a marked unresponsiveness to external stimuli or dullness is observed (Smart and Charlton, 1992). It is not known to what degree the distribution of rabies virus in the CNS might be correlated to these clinical findings. Thus, the objectives of this experiment were fourfold: i) to determine the pattern of skunk street rabies virus distribution in early stage rabies in the CNS of rabid skunks, ii) to identify regions associated with heavy virus accumulations, iii) to determine the degree of variation in virus distribution between

experimental animals, and iv) to determine if biting attacks are a reliable indicator of early stage furious rabies in rabid skunks.

In order to achieve these objectives, the method by which virus or viral antigen is detected in tissue sections is of paramount importance. Two methods are currently in use for the detection of rabies virus antigen in nervous tissues. Although immunofluorescence techniques are most commonly used for confirmation of rabies virus infection in diagnostic specimens, the immunoperoxidase staining technique offers some obvious advantages for studying experimental rabies infections. Due to the preservation of anatomical detail in immunoperoxidase treated sections, the location and density of rabies virus antigen can be specifically mapped to individual nuclei and/or regions within the CNS.

Immunoperoxidase studies of the distribution of rabies virus antigen in the CNS, during experimental or naturally occurring infection are few. Studies of the distribution of fixed virus such as CVS virus (Jackson and Reimer, 1989; Jackson, 1990) or street virus in terminal cases submitted for rabies diagnosis (Feiden et al., 1985; Feiden et al., 1987), are not sufficient to elucidate the pattern of rabies virus distribution in the CNS during the early stages of naturally occurring infection.

The experiments described in this and subsequent chapters are basic to the study of rabies aggressive behavior since

they were done in a species that supports enzootic rabies in nature and detailed determinations of antigen distribution were done early in the period of clinical signs (when the difference between furious and dumb rabies is most apparent).

Experimental design

Eight skunks (4 males, 4 females) were inoculated intramuscularly with skunk street virus ($10^{7.0}$ TCID₅₀/ml) and 2 skunks were inoculated with diluent only. Skunks were observed at least twice daily for clinical signs of rabies. When they would attack and bite a stick placed within their visual range or were at least mildly paretic they were humanely killed. The brains were removed, immersion fixed, and processed as previously described in Chapter 2. Two 5 micron adjacent sections were cut at 100 micron intervals and one was stained by the ABC immunoperoxidase technique and the other was stained with cresyl violet acetate. A piece of spinal cord was submitted to the A.D.R.I. rabies diagnostic laboratory for antibody confirmation of rabies virus infection by immunofluorescence.

Results

All of the skunks inoculated with street rabies virus were diagnosed positive for rabies as determined by the fluorescent antibody test. The clinical signs exhibited by these animals have been summarized in Table 2. In general,

Table 2. Clinical signs in skunks infected with skunk street rabies virus at time of euthanasia.

| Skunk number | 1043 | 1045 | 1047 | 1049 | 1050 | 1052 | 1054 | 1056 |
|-----------------------|-----------------|------|----------------|------|------|------|------|------|
| Incubation | 34 ^a | 18 | 18 | 19 | 18 | 18 | 19 | 18 |
| Disease course | 1 | 2 | 2 | 1 | 1 | 2 | 2 | 2 |
| Death | E ^b | E | E | D | E | E | E | E |
| Ataxia | ND ^c | ND | - ^d | ND | ND | - | + | + |
| Paresis ^e | - | + | - | ND | - | + | - | - |
| Stick test | +++ | - | +++ | ND | +++ | + | +++ | +++ |
| Recumbency | - | - | - | - | - | - | - | - |
| Dullness | - | + | - | - | - | + | - | - |
| Rigidity ^f | - | - | - | - | - | - | - | - |
| Tremors | - | - | - | - | - | - | - | - |
| Hyperactivity | ++ | - | ++ | ++ | ++ | - | ++ | ++ |

a) in days, for both incubation and disease course

b) death by euthanasia (E), found dead in cage (D)

c) ND - not determined

d) Grading marks, - not observed, + mild, ++ moderate, +++ marked clinical signs

e) Paresis of hind limbs

f) Rigidity of extensor muscles

the clinical progression of the disease was slow with only mild advancement of clinical signs until the time of sacrifice, except for skunk 1049 which died suddenly after showing clinical signs for only one day. Post mortem autolysis rendered tissues from this skunk unsuitable for further immunoperoxidase staining and interpretation. For 7 of the 8 skunks, incubation times were 18 or 19 days while skunk 1043 had an incubation period of 34 days. The initial signs of rabies were hyperactivity and a positive stick test (illustrated in Figure 1), except for skunks 1045 and 1052 which exhibited dullness and mild paresis. If the initial signs were only mild on the first day of observation of clinical signs, the disease was allowed to progress one more day before the animals were humanely killed. The two control animals did not develop rabies during the 45 day observation period.

The results of immunoperoxidase staining are summarized in Table 3. Rabies virus antigen was detected in all of the structures listed. It was visualized either as brown dust-like particles or as larger dark brown to black discrete granules in the cytoplasm of neurons. Staining was present in mild, moderate, marked or very marked amounts (Figure 2).

Figure 1

Figure 1. Biting behavior of an experimentally infected rabid skunk.

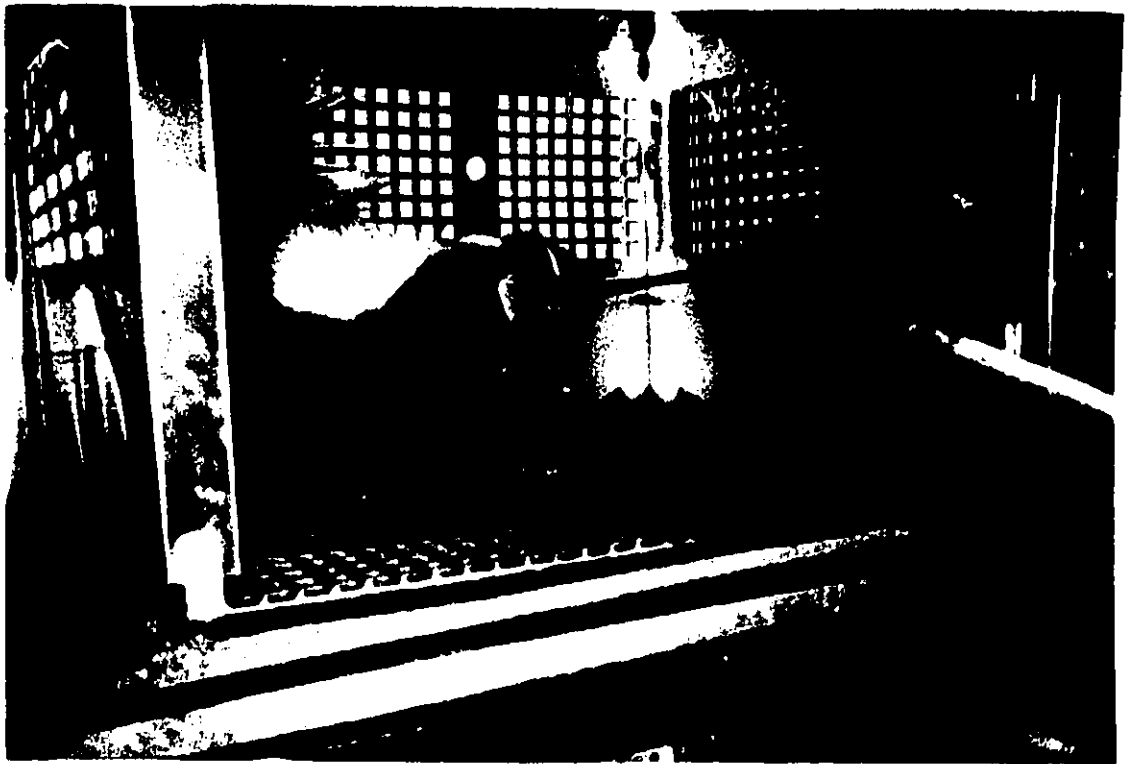


Table 3. Location and intensity of immunoperoxidase staining of rabies virus antigen in the CNS of intramuscularly infected rabid skunks.

| Skunk number | 1043 | 1045 | 1047 | 1050 | 1052 | 1054 | 1056 |
|------------------------------|------|------|------|------|------|------|------|
| Gracile n. | +++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Cuneate n. | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Inferior olivary n. | ++ | ++ | + | + | + | + | + |
| Accessory cuneate n. | + | +++ | + | + | + | + | + |
| Spinal n. of V | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Dorsal motor n. of X | ++ | +++ | ++ | +++ | +++ | ++ | ++ |
| Hypoglossal n. | +++ | ++ | +++ | ++ | ++ | +++ | ++ |
| Abducens n. | + | + | ++ | ND | ++ | + | + |
| Cochlear n. | + | +++ | ++ | + | + | ++ | ++ |
| Facial n. | +++ | +++ | +++ | +++ | ++ | ++++ | +++ |
| Motor n. of V | +++ | ++++ | +++ | +++ | +++ | +++ | +++ |
| Vestibular nuclei | ++ | +++ | ++ | ++ | ++ | +++ | ND |
| Trochlear n. | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Superior olivary n. | ++ | ++ | + | + | + | + | + |
| Nuclei pontis | + | ++ | + | ++ | + | + | + |
| Purkinje cells | + | + | + | + | + | + | + |
| Granular layer | + | + | + | + | + | + | + |
| Deep cerebellar n. | + | ++ | ++ | + | + | ++ | + |
| Red nucleus | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Substantia nigra | ++ | ++ | ++ | ++ | ++ | +++ | +++ |
| Medullary raphe n. | +++ | ++ | +++ | ++ | ++ | +++ | ++ |
| Pontine raphe n. | ++ | ++ | +++ | ++ | +++ | ++ | ++ |
| Midbrain raphe n. | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Dorsal raphe n. ^a | +++ | +++ | +++ | +++ | ++ | ++ | ++ |
| Medulla reticular n. | +++ | ++++ | +++ | ++ | +++ | +++ | +++ |
| Pontine reticular n. | ++ | ++++ | ++ | ++ | +++ | +++ | ++ |
| Mesencephalic n. of V | ++ | ++ | + | ++ | ++ | ++ | ++ |
| Superior colliculus | + | + | + | + | + | + | + |
| Inferior colliculus | + | + | + | + | + | + | + |
| Interpeduncular n. | ++ | + | + | + | + | + | + |
| Ventral periaqueductal grey | ++ | ++ | +++ | +++ | +++ | +++ | ++ |
| Dorsal periaqueductal grey | + | + | + | + | + | + | + |
| Oculomotor nuclei | ++ | + | ++ | ++ | ++ | ++ | ++ |
| Pulvinar | + | + | + | + | + | + | + |
| Pineal body | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Habenular nuclei | + | ND | + | + | + | + | + |
| Hippocampal formation | ++ | ND | +++ | ++ | ++ | ++ | + |
| Dentate gyrus | + | ND | ++ | ++ | ++ | + | + |
| Thalamus | + | + | + | + | + | + | + |
| Nucleus subthalamicus | ++ | ++ | + | ++ | + | + | + |
| Hypothalamus | ++ | ++ | ++ | ++ | ++ | ++ | ND |

Table 3. (continued)

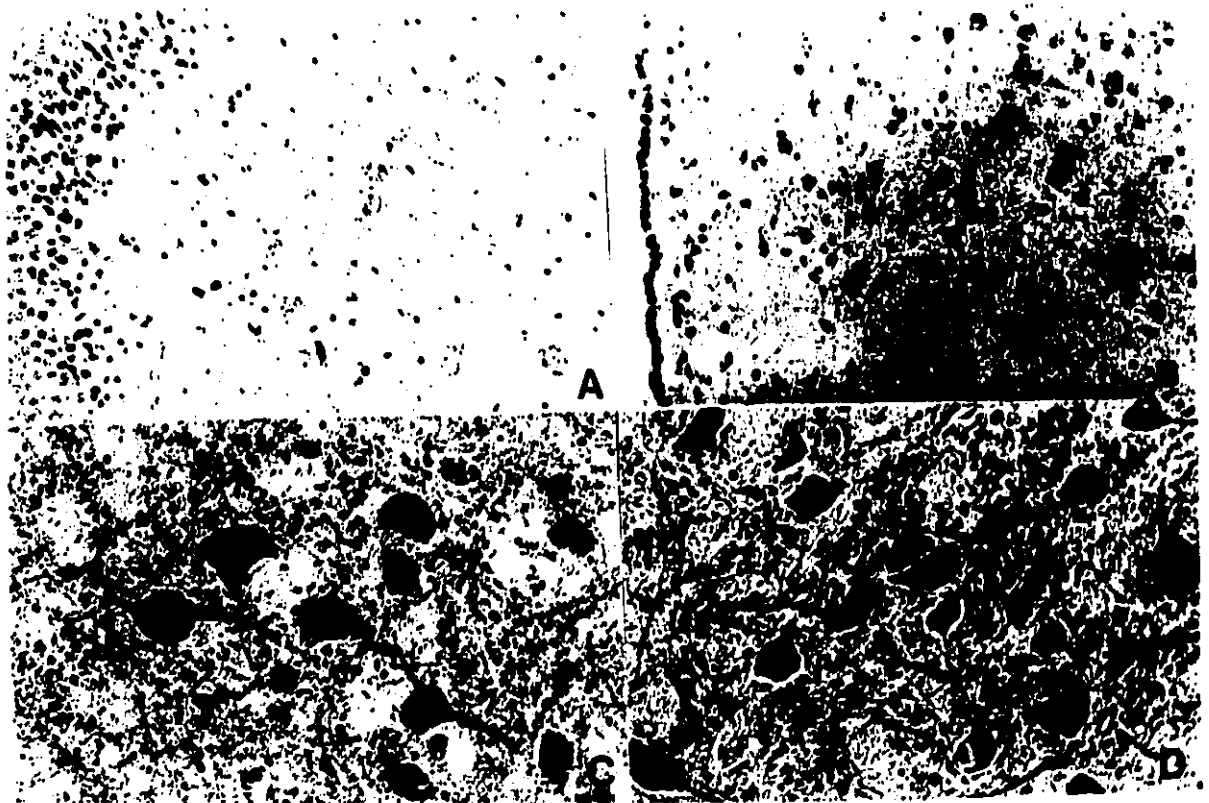
| | | | | | | | |
|------------------------------|----|---|---|----|----|----|----|
| Mamillary bodies | + | + | + | + | + | + | + |
| Pyriiform lobe of cortex | + | + | + | ++ | + | ++ | + |
| Amygdaloid n. | ++ | + | + | ++ | + | + | + |
| Caudate n. | + | + | + | + | + | + | + |
| Septal nuclei | ++ | + | + | + | + | + | + |
| Cortex except pyriiform lobe | + | + | + | + | + | + | + |
| Olfactory lobe | + | + | + | + | ND | + | ND |

n. - nucleus

a Rostral margin, at the level of the oculomotor nuclei.

ND - not determined, 0 - no antigen detected, +, ++, +++, +++, mild, moderate, marked or very marked accumulations of antigen, respectively.

Figure 2. Examples of grading marks used for describing the intensity of immunoperoxidase staining of rabies virus antigen; mild, moderate, marked and very marked. A) mild accumulation of viral antigen in the region of the dentate gyrus, B) moderate accumulation of viral antigen in the hypothalamus, C) marked accumulation of viral antigen in the motor nucleus of the trigeminal and D) very marked accumulation of viral antigen in the facial nucleus.
(MAG, A,B,C,D 400X)



Since intensity of staining should correlate with the concentration of virus antigen, it can be seen that there was considerable variation in the antigen content of different nuclei or regions (Table 3). Accumulation of antigen was mild to moderate in most regions of the medulla oblongata except for the hypoglossal, dorsal motor nucleus of the vagus, vestibular nuclei and medullary reticular neurons which contained marked accumulations of antigen (Table 3). In the pons, marked accumulation of virus was found in the pontine raphe nuclei, and reticular neurons. The midbrain raphe nuclei (including the dorsal raphe nucleus), ventral periaqueductal grey and red nucleus also contained a marked amount of virus. Virus accumulation in structures rostral to the midbrain was mild to moderate. While there was variation in the quantity of antigen in the regions of the brain of the infected skunks, there was remarkable similarity in the distribution of viral antigen between skunks. This consistency was also evident for skunk 1043 in spite of the 34 day incubation period.

Discussion

This experiment provides further support for the hypothesis that rabies virus accumulates preferentially in selected CNS structures. Although virus was inoculated peripherally and most likely gained access to brainstem structures via the spinal cord, many of the regions of this

area such as cerebellum and accessory cuneate nucleus, contained only small amounts of virus. This is in contrast to more rostral regions such as the midbrain raphe nuclei and red nucleus in which large quantities of viral antigen were demonstrated. Although rabies virus has been shown to spread in the CNS via functionally connected areas (Kucera et al., 1985; Gillet et al., 1986), the route of rabies virus spread through the CNS has not been fully described. It is possible that rabies virus fails to replicate (and thus accumulate) in some of the regions it migrates through or that several potential pathways for virus spread are present. The basis for this selective type of neurotropism is completely unknown but cell receptor or neurotransmitter mechanisms have been proposed (Lentz et al., 1988; Tsiang, 1988).

The correlation between the clinical signs of furious rabies and accumulations of virus within the CNS is speculative at this time. In some other neurotropic infectious, such as Borna disease, neuronal cell damage often correlates to the pattern of viral infection (Stitz et al., 1989). However, neuronal degeneration is uncommon in rabies infected CNS, despite the bizarre behavioral changes of rabid animals. Thus it has been proposed that an imbalance in luxury function such as neurotransmitter activity may account for the clinical signs of rabies.

In this experiment, we observed that the greatest amount of virus antigen was present in the facial, trigeminal (motor)

and red nuclei, and in reticular and raphe neurons of the medulla, pons and midbrain as well as the ventral periaqueductal grey matter. Most of these regions are presently implicated in pathways of biting behavior in the cat and other species (Bandler, 1988; Seigel and Brutus, 1990). Ablation or dysfunction of portions of the ventral periaqueductal grey matter or regions containing midbrain raphe nuclei has been shown to lead to an increase in aggression and biting behavior in many animal models and human studies (Yamamoto and Ueki, 1977; Soubrie, 1986). For example, the raphe nuclei of the medulla, pons and midbrain, contain the majority of the serotonin present in the CNS. Serotonin is well described as a neurotransmitter which is associated with the inhibition of excitability and aggressive impulses (Valzelli, 1984; Soubrie, 1986). Thus, it might be suggested that the accumulation of rabies virus in serotonin-containing neurons may lead to a reduction of the serotonin influence in the CNS, which then might facilitate the aggressive outbursts associated with furious rabies.

It is unclear why two skunks in this experiment (1045, 1052), did not exhibit biting behavior, despite a distribution of rabies virus similar to the other skunks in this study. To clarify this finding, the next experiment was designed to further investigate the pattern of rabies virus distribution in the CNS of biting and non-biting rabid skunks.

CHAPTER 4

The distribution of Challenge Virus Standard rabies virus as compared to skunk street rabies virus in the brains of experimentally infected rabid skunks.

Introduction

Although aggressive behavior or biting attacks have been the hallmark of rabies throughout the centuries (Wilkinson, 1988), the dumb form of rabies also occurs, in which aggressive outbursts are conspicuously absent. Whether or not these 2 different syndromes might arise due to differences in the distribution of rabies virus in the CNS of infected animals (Johnson, 1965; Jackson and Reimer, 1989), or as a result of the effects of immunopathology due to the host response to rabies virus (Iwasaki et al., 1977; Hemachuda et al., 1988; Sriwanthana et al., 1989) is still a matter of some controversy (see Chapter 1 for a more detailed discussion). This study is restricted to investigation of the former possibility.

In 1965, Johnson proposed that rabies virus tropism for neurons in the limbic system, as visualized by the fluorescent antibody technique, might explain the emotive outbursts of rabid animals. Several recent studies involving experimental infections with fixed virus in mice (Jackson and Reimer, 1989; Jackson, 1991), have given results that are not entirely consistent with Johnson's (1965) earlier claims. To further

investigate the distribution of rabies antigen in the CNS, in the early stages of rabies, skunks were inoculated with either CVS virus or skunk street rabies virus. In our laboratory, skunks inoculated with skunk street virus exhibit predominantly furious rabies characterized by biting attacks while skunks infected with CVS virus show paralytic clinical signs consistent with dumb rabies. It was necessary to use CVS virus to produce dumb rabies because street rabies virus infection of skunks rarely and unpredictably results in dumb rabies. Thus in order to minimize the number of experimental animals needed, the former approach was selected for this experiment. The objectives of this experiment were to determine the pattern of rabies virus antigen accumulation in the CNS following inoculation of CVS virus (dumb rabies) or skunk street rabies virus (furious rabies) during early stage rabies and to determine if the route of inoculation is associated with virus distribution for skunk street virus.

Experimental design

The details of the following procedures are described in Chapter 2. Sixteen skunks were divided into two groups each having equal numbers of male and female skunks. One group received CVS virus and the other group received skunk street virus. Two skunks served as uninfected controls receiving the respective diluents only. Skunks were inoculated intranasally with 0.5 ml of either the 10% skunk salivary gland suspension

(street virus, $10^{8.5}$ TCID50/ml) or CVS rabies virus ($10^{8.5}$ TCID50/ml). Intranasal inoculation was used for this trial because intramuscular inoculation with CVS rabies virus rarely results in clinical rabies.

Skunks were humanely killed when they either attacked a stick placed within their range or were at least mildly dull and/or paretic. Brains were removed, immersion fixed, processed, sectioned, stained and evaluated as previously described in Chapter 2.

Results

Clinical data

All of the skunks that became clinically ill in these experiments were rabies positive by the fluorescent antibody test. Two skunks inoculated intranasally with street virus and the 2 control skunks did not develop rabies. The incubation periods were similar for skunks of the same experimental group (Table 4). Skunks inoculated with CVS virus had a shorter incubation time of 7 - 8 days, while skunks inoculated with street virus did not show clinical signs until 12 - 14 days post inoculation. The first sign of rabies for most of the street virus infected skunks was hyperresponsiveness to stimuli such as noise and touch, and immediate attack of a stick presented through the bars of the cage. Some of these skunks had mild inco-ordination and paresis associated with the hind limbs but were still able to

Table 4. Clinical signs in skunks infected with street or CVS rabies virus at time of euthanasia.

Street virus

| Skunk number | 1224 | 1227 | 1229 | 1232 | 1233 | 1326 |
|-----------------------|-----------------|------|------|------|------|------|
| Incubation | 13 ^a | 12 | 12 | 14 | 14 | 12 |
| Disease course | 1 | 1 | 2 | 1 | 1 | 1 |
| Death | E ^b | E | E | E | E | E |
| Ataxia | - ^c | + | + | - | - | - |
| Paresis ^d | - | - | - | - | - | - |
| Stick test | ++ | +++ | ++ | ++ | ++ | +++ |
| Recumbency | - | - | - | - | - | - |
| Dullness | - | - | - | - | - | - |
| Rigidity ^e | - | - | - | - | - | - |
| Tremors | - | - | - | - | - | - |
| Hyperactivity | ++ | ++ | ++ | ++ | ++ | ++ |

CVS virus

| Skunk number | 1217 | 1221 | 1225 | 1291 | 1293 | 1295 | 1297 | 1298 |
|----------------|------|------|------|------|------|-----------------|------|------|
| Incubation | 7 | 8 | 8 | 7 | 7 | 7 | 7 | 8 |
| Disease course | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Death | E | E | E | E | E | E | E | E |
| Ataxia | + | + | + | + | + | ND ^f | + | + |
| Paresis | + | - | + | + | - | ND | ND | - |
| Stick test | + | - | - | + | - | - | - | - |
| Recumbency | - | + | - | - | - | + | - | - |
| Dullness | + | + | + | + | - | + | + | + |
| Rigidity | ++ | +++ | - | + | + | + | ++ | ++ |
| Tremors | - | - | - | - | + | + | - | - |
| Hyperactivity | - | - | - | - | - | - | - | - |

a) in days, for both incubation and disease course

b) death by euthanasia

c) Grading marks, - not observed, + mild, ++ moderate, +++ marked clinical signs

d) Paresis of hind limbs

e) Rigidity of extensor muscles

f) ND = not determined

move freely and quickly in response to stimulation. These animals were able to consume food at this stage, and excess salivation was not a predominant clinical feature.

All skunks inoculated intranasally with CVS virus developed rabies. The first clinical sign noted in these skunks was instability and inco-ordination of the hind limbs. These animals were dull and unresponsive to external stimulation and some exhibited an intention tremor and/or extensor rigidity associated with attempted locomotion. A few skunks progressed to lateral recumbency within 24 hours of onset of clinical signs. Skunks 1217 and 1291 had a very mild response (investigation and tentative chewing) to the stick test, but this was quite distinct from the manic destruction of the stick exhibited by street virus infected skunks.

Virus distribution

Rabies virus antigen was detectable in all the structures listed in Table 5. The amount contained within neurons varied considerably between structures. Antigen was visualized as either fine brown dust-like particles scattered throughout the cell bodies of neurons, and/or as a collection of small to large dark brown or black granules present in cell bodies, and fibres, (mainly dendrites with lesser amounts in axons). As found in the previous experiment, the location and density of rabies antigen was similar between animals of the same experimental group. For this reason, the distribution of

Table 5. Distribution of street rabies virus as compared to CVS rabies virus in the CNS of intranasally infected rabid skunks.

| Brain region/nucleus | Skunk street ^a | CVS ^b |
|------------------------------------|---------------------------|------------------|
| Gracile n. ^c | ++ | ++ |
| Cuneate n. | ++ | ++ |
| Inferior olivary n. | ++ | ++ |
| Accessory cuneate n. | ++ | ++ |
| Spinal n. of the trigeminal | ++ | ++ |
| Dorsal motor n. of the vagus | +++ | ++ |
| Hypoglossal n. | +++ | + |
| Abducens n. | +++ | ++ |
| Cochlear n. | ++ | +++ |
| Facial n. | +++ | + |
| Motor n. of the trigeminal | +++ | + |
| Vestibular nuclei | +++ | +++ |
| Trochlear n. | +++ | ++ |
| Superior olivary n. | + | ++ |
| Nuclei pontis | ++ | ++ |
| Purkinje cells | + | ++++ |
| Granular layer, cerebellum | + | + |
| Fastigial n. | +++ | +++ |
| N. interpositus | +++ | +++ |
| Dentate n. | +++ | +++ |
| Red n. | +++ | + |
| Substantia nigra | +++ | +++ |
| Medullary raphe nuclei | +++ | +++ |
| Pontine raphe nuclei | +++ | ++ |
| Midbrain raphe nuclei | +++ | +++ |
| Dorsal raphe n.^d | +++ | + |
| Medulla reticular nuclei | +++ | +++ |
| Pontine reticular nuclei | +++ | +++ |
| Mesencephalic n. of the trigeminal | ++ | +++ |
| Parapeduncular n. | + | + |
| Superior colliculus | + | ++ |
| Inferior colliculus | + | ++ |
| Interpeduncular n. | + | ++ |
| Ventral periaqueductal grey | +++ | +++ |
| Dorsal periaqueductal grey | + | + |
| Oculomotor nuclei | ++ | + |
| Pulvinar | + | + |
| Pineal body | + | + |
| Habenular nuclei | + | +++ |
| Hippocampal formation | +++ | +++ |
| Dentate gyrus | ++ | ++ |
| Thalamus | + | ++ |
| Nucleus subthalamicus | ++ | +++ |
| Hypothalamus | +++ | ++ |

Table 5. (continued)

| | | |
|-------------------------------------|----------|------------|
| Mamillary bodies | + | + |
| Pyriiform lobe of cortex | ++ | ++ |
| Amygdaloid nucleus | ++ | +++ |
| Caudate nucleus | + | ++ |
| Septal nuclei | + | + |
| Cortex except pyriiform lobe | + | +++ |
| Olfactory lobe | + | +++ |

a) average for 6 skunks

b) average for 8 skunks

c) n. - nucleus

d) Rostral margin, measured at the level of the oculomotor nuclei. Posterior to this region, street or CVS virus is present in marked amounts in cell bodies and fibres.

Grading: +, ++, +++, +, mild, moderate, marked, or very marked accumulations of antigen, respectively.

Areas in bold are summarized in Table 6

antigen shown in Table 5 is the average observed for all animals of each experimental group. There were some obvious differences in virus antigen distribution between the street and CVS virus infected animals as summarized in Table 6. Areas which contained heavy accumulations of street rabies virus antigen but very low amounts of CVS virus antigen were the neurons and cell processes of the dorsal motor nucleus of the vagus, hypoglossal, dorsal raphe nucleus at the level of the oculomotor nucleus and red nuclei. In contrast, large accumulations of CVS virus antigen were found in the Purkinje cells of the cerebellum, the habenular nuclei and in pyramidal cells throughout the cerebral cortex, while corresponding areas in all street virus infected skunks contained little antigen (Figures 3 and 4). Table 5 also shows that other areas had smaller differences between amounts of street and CVS virus accumulations present in various regions of the CNS.

Table 6. Summary of the differences in antigen distribution in defined areas of the CNS of rabid skunks between CVS rabies virus and skunk street rabies virus.

Street virus

| | XII | VII | Purk | Drn | Thal | Hab | Red | Cortex | Olf |
|------|-----|-----|------|-----|------|-----|-----|--------|-----|
| 1224 | ND | ++ | + | +++ | + | + | +++ | + | ND |
| 1227 | ND | ND | ++ | + | + | + | +++ | + | ND |
| 1229 | +++ | +++ | + | ++ | + | + | +++ | + | + |
| 1232 | ++ | +++ | + | ++ | + | + | +++ | + | + |
| 1233 | ++ | +++ | + | +++ | + | + | +++ | + | + |
| 1326 | +++ | +++ | + | +++ | + | + | +++ | + | + |

CVS virus

| | | | | | | | | | |
|------|----|----|------|-----|-----|-----|---|------|-----|
| 1217 | + | + | ++++ | +++ | +++ | +++ | + | ++++ | +++ |
| 1221 | + | + | ++++ | + | ++ | +++ | + | +++ | +++ |
| 1225 | ND | ND | ++++ | 0 | + | +++ | + | ++++ | ND |
| 1291 | + | + | +++ | + | ++ | ND | + | +++ | ND |
| 1293 | + | + | +++ | +++ | ++ | +++ | + | ++++ | ND |
| 1295 | + | + | +++ | + | + | +++ | + | +++ | +++ |
| 1297 | ND | ND | ++++ | + | ++ | +++ | + | +++ | ND |
| 1298 | + | + | +++ | 0 | +++ | +++ | + | +++ | +++ |

XII - hypoglossal nucleus, VII - facial nucleus, Purk - purkinje cells and dendrites in the molecular layer of cerebellum, Drn - dorsal raphe nucleus, Thal - thalamus including, medial and lateral geniculate bodies, Hab - habenular nuclei, Red - red nucleus, Cortex - cerebral cortex, Olf - olfactory lobe. ND - not determined.

Grading marks: 0, no antigen detected; +, ++, +++, +++++, mild, moderate, marked or very marked accumulations of antigen, respectively; ND - not determined.

Fig. 3 A-D. Immunoperoxidase-hematoxylin stained sections of the Purkinje cells of the cerebellum (A and B) and red nucleus (C and D). CVS rabies virus is detectable in large amounts in the Purkinje cells of the cerebellum in A and is barely detectable in the red nucleus (D). Street rabies virus is present in large amounts in the red nucleus (C), but is barely detectable in the Purkinje cells of the cerebellum in B. (MAG, A,B,C,D, 400X)

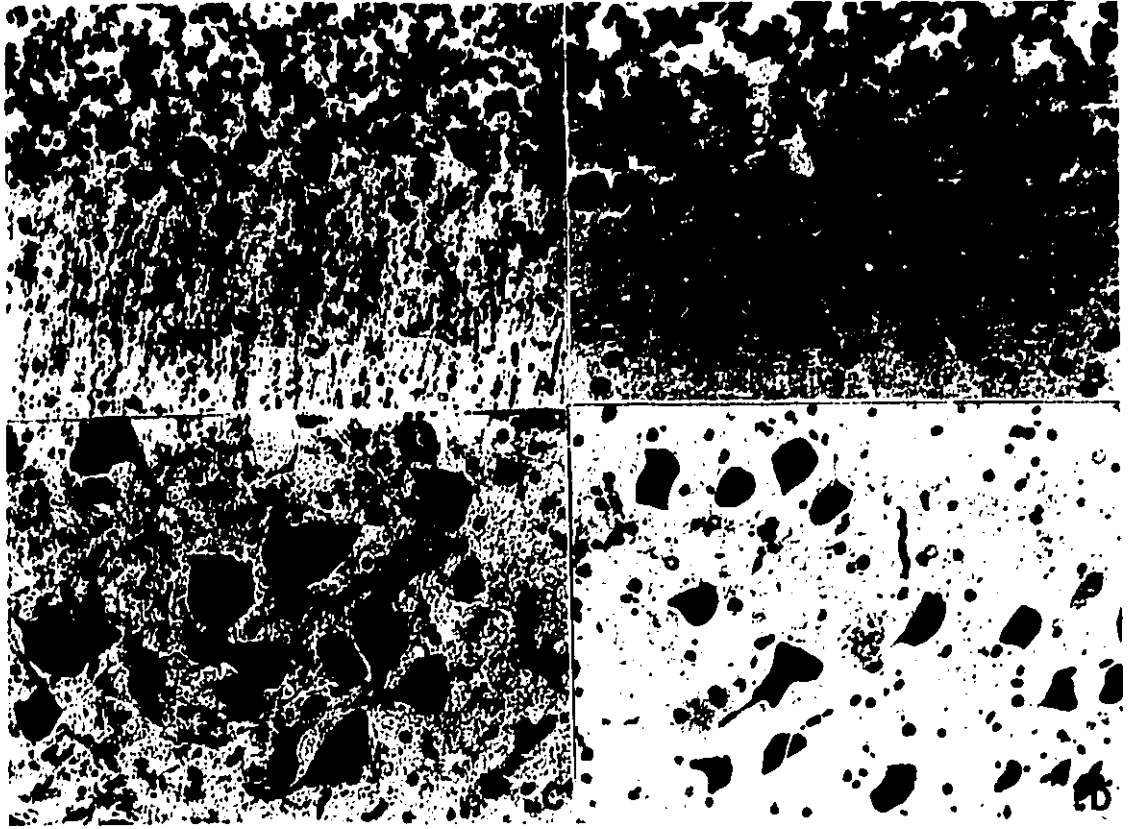
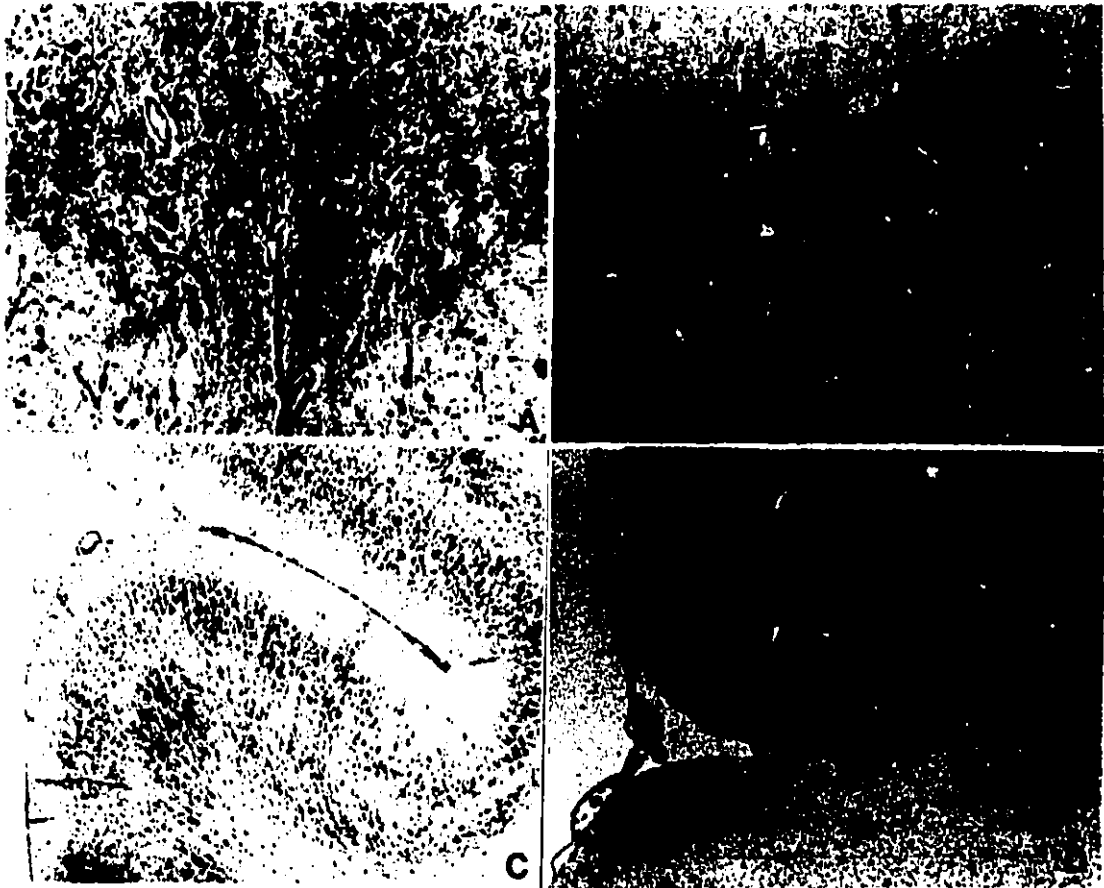


Fig. 4 A-D. Immunoperoxidase-hematoxylin stained sections of the dorsal raphe nucleus at the level of the oculomotor nucleus (A and B) and cerebral cortex (C and D). CVS rabies virus is present in large amounts in the pyramidal cells of the parietal cerebral cortex (C) and but not in the dorsal raphe nucleus (B). Street rabies virus is present in small amounts in the cerebral cortex (D) and in marked amounts in the dorsal raphe nucleus (A). (MAG, A,B,C,D, 400X)



Discussion

This experiment demonstrates marked differences in the distribution of fixed versus street rabies virus antigen in the brains of experimentally infected skunks during early stage rabies. This is in contrast to the diffuse presence of rabies virus antigen in the terminal stages of naturally occurring and experimentally induced disease (Feiden et al., 1988). Although the extent to which the visualization of antigen represents intact virus particles is unknown, it is assumed that the intensity of the anti-RNP staining is an indirect measure of the number of replicating and complete virions produced in the neuron.

Factors which have been shown to affect various aspects of rabies pathogenesis include; route of inoculation (Fekadu et al., 1982; Feiden et al., 1988; Jackson and Reimer, 1989), dose (Dietzschold et al., 1985; Jackson, 1991), viral strain (Tsiang and Guillon, 1981), immunocompetence (Iwasaki et al., 1975; Hemachuda et al., 1988) and species of the host (Baer, 1975; Charlton, 1988), but not all of these have been shown to influence the distribution of virus in the CNS. This experiment has shown that the strain of virus inoculated has a significant effect both on the distribution of virus antigen in the CNS and on the clinical course of the disease. In fact, the variation in the location and amount of virus antigen between CVS and skunk street rabies virus suggests that the pathogenesis of rabies may be different for these two

viral strains. The following factors, as reported in the literature, seem to be less important. It has been shown that infection with reduced dose inocula or the use of apathogenic mutants, results in slower viral replication but no differences in viral distribution within the CNS at the time of clinical rabies (Dietzschold et al., 1985; Jackson, 1991). Charlton et al., (1984) demonstrated that immunosuppression of skunks experimentally infected with skunk street virus did not alter the clinical course of the disease. These observations also support the possibility that the multiplication and dispersal of the virus in the CNS follows a particular virus determined tropism, the basis for which is still unknown.

In this study, we speculate that there is a functional significance of the heavy concentration and specific accumulation of street versus CVS virus antigen. Since antigen is present in the CNS prior to clinical signs (Johnson, 1965; Jackson and Reimer, 1989), it is possible that a threshold amount of virus must be exceeded within neurons before the clinical effects of viral infection are evident. Several areas in which differences in the amount of antigen accumulation were identified in this experiment, are known to be involved in behavior or locomotor pathways. For example, the large amount of CVS virus antigen in the Purkinje cell layer of the cerebellum may contribute to the hypermetria, inco-ordination and recumbency so characteristic of fixed virus infection of skunks. In street virus infection, we

postulate that the presence of virus antigen in the midbrain raphe nuclei, at the level of the oculomotor nucleus (a region shown to inhibit aggressive behavior by the production of the neurotransmitter serotonin (Seigel and Brutus, 1990; Yamamoto and Ueki, 1977)), is necessary for the display of biting behavior seen in this group of skunks.

Although the effect of rabies virus on neuronal cell physiology is unknown, it is possible that neurons which contain greater quantities of virus are more likely to dysfunction. However, rabies infected neurons in the CNS rarely show light or electron microscopic evidence of damage. Thus, in order to elucidate the mechanism for the behavioral abnormalities associated with rabies disease, it is necessary to select an indirect measure of cell function. It has been proposed that rabies causes a reduction in the amount of neurotransmitter produced by infected neurons (Tsiang, 1988; Gourmelon, 1991). Thus, the following experiment was designed to determine if street rabies and/or CVS or fixed rabies infection causes a reduction in the levels of serotonin and met-enkephalin (as determined by the intensity of immunoperoxidase staining), in neurons and fibres of the CNS of rabid skunks.

CHAPTER 5

The effect of skunk street rabies virus and CVS rabies virus on the intensity of serotonin and met-enkephalin immunoperoxidase staining in the CNS of experimentally infected rabid skunks.

Introduction

The pathogenesis of the behavioral changes associated with rabies has not yet been described. Despite the bizarre behavioral signs of rabid animals, rabies infected CNS neurons rarely show light or electron microscopic evidence of damage. This enigma has led to the notion that rabies may be a disease of impaired information transmission. Gourmelon et al., 1986; Tsiang, 1988; and Gourmelon et al., 1991 have proposed that rabies virus infection of neurons may interfere with an as yet unidentified aspect of neurotransmitter metabolism. However, since Johnson (1965) speculated that rabies virus infects a select group of neurons in the CNS (leading to neuronal dysfunction), further support for his theory has not been demonstrated.

In the previous experiment, it was shown that biting attacks were associated with skunk street virus rabies, and that these outbursts were absent in skunks infected with CVS rabies virus. It was also shown that skunk street virus but not CVS rabies virus was present in marked amounts in the midbrain raphe nuclei at the level of the oculomotor nucleus,

a region implicated in the control of aggressive behavior (Pucilowski and Kostowski, 1983; Soubrie, 1986). The midbrain raphe nuclei are known to secrete the inhibitory neurotransmitters serotonin and met-enkephalin (Steinbusch, 1983; Stewart, 1989). Reduction(s) in the level of inhibitory neurotransmitter(s), may be a mechanism which facilitates an increase in the level of aggressive behavior of rabid skunks.

The objectives of this experiment were to determine if a reduction in the levels of serotonin and/or met-enkephalin could be detected in rabies infected CNS by anti-serotonin and anti-met-enkephalin immunoperoxidase staining and to determine if rabies virus selectively accumulates in serotonergic and/or met-enkephalinergic cell bodies and processes.

Experimental Design

Ten skunks of approximately one year of age were divided into the following groups. Two skunks (one male, one female) served as uninoculated controls; four skunks (two male, two female) received CVS rabies virus; four skunks (two male, two female) received skunk street virus. Each animal received 0.5 ml of either 10% salivary gland suspension ($10^{7.5}$ TCID₅₀/ml or CVS rabies virus $10^{7.5}$ TCID₅₀/ml) intranasally. The control animals received 0.5 ml of one of the respective diluents administered intranasally.

When the skunks were judged to be showing early clinical signs of rabies, they were perfused with 4% paraformaldehyde

pH 7.3. The brains were removed and fixed in perfusant overnight at room temperature. The tissues were blocked, dehydrated, and embedded in paraffin wax using routine histological procedures. The brains were serially sectioned from caudal medulla to the rostral margins of the cerebral cortex and every 100 microns three sections were saved and mounted on gelatin coated glass Probe-on slides (Fischer). One section was subjected to anti-rabies RNP immunoperoxidase staining and then evaluated in exactly the same manner as described for the previous experiment (Chapter 4). The other two sections were subjected to either anti-serotonin or anti-met-enkephalin immunoperoxidase staining. Negative control slides were subjected to the immunoperoxidase staining protocol but the primary antibody was omitted from the first incubation. Each staining run included brain tissues infected with street rabies virus, with CVS rabies virus and positive and negative control sections. The location and density of immunoperoxidase staining for rabies and as well as serotonin and met-enkephalin was compared between skunk street rabies virus as compared to CVS rabies virus in selected areas throughout the brain.

Results

Clinical data

All of the skunks inoculated with either skunk street or CVS rabies virus developed rabies, as confirmed by anti-rabies

RNP immunoperoxidase staining. The two control skunks remained healthy until the completion of the observation period. The incubation periods were similar for skunks of the same experimental group. They ranged from 8 to 9 days for the CVS rabies virus infected group and 13 to 18 days for the skunk street virus infected skunks (Table 7). As with the trials described in Chapters 3 and 4, the first sign of rabies in the skunks infected with skunk street rabies virus were hyperactivity and biting of a stick placed within their range. The CVS virus infected skunks were extremely dull and unresponsive to touch and/or noise and several of these quickly progressed to lateral recumbency within 24 hours after the onset of clinical signs. Only the recumbent animals showed mild to moderate evidence of excessive salivation.

Virus distribution

The location of rabies virus antigen in the CNS in both the CVS and the skunk street rabies virus groups was evaluated as described for the previous experiment (Chapter 4). However, a reduction in the intensity of immunoperoxidase staining was observed due to the use of paraformaldehyde as a fixative (personal communication, A. Bourgon, 1991). Although optimum immuno-peroxidase detection of rabies virus in paraffin embedded tissues is achieved with formaldehyde pH 5.3 fixation (Bourgon and Charlton, 1987), paraformaldehyde fixation was required for immunoperoxidase staining of

Table 7. Clinical signs in skunks infected with street rabies virus or CVS rabies virus, (for neurotransmitter studies) at time of euthanasia.

Street virus

| Skunk number | 1568 | 1574 | 1578 | 1579 | 1587 |
|-------------------------|----------------|------|------|------|------|
| Incubation ^a | 18 | 13 | 17 | 13 | 14 |
| Disease course | 2 | 2 | 1 | 1 | 2 |
| Death | E ^b | E | D | E | E |
| Ataxia | - ^c | - | - | - | - |
| Paresis ^d | - | - | - | - | - |
| Stick test | +++ | +++ | +++ | +++ | +++ |
| Recumbency | - | - | - | - | - |
| Dullness | - | - | - | - | - |
| Rigidity ^e | - | - | - | - | - |
| Tremors | - | - | - | - | - |
| Hyperactivity | +++ | +++ | +++ | +++ | +++ |

CVS virus

| Skunk number | 1564 | 1576 | 1581 | 1583 | 1585 |
|----------------|------|------|------|------|------|
| Incubation | 8 | 9 | 9 | 8 | 8 |
| Disease course | 1 | 1 | 1 | 1 | 1 |
| Death | E | E | E | E | E |
| Ataxia | - | ND | - | ND | - |
| Paresis | - | ND | - | ND | - |
| Stick test | - | - | - | - | - |
| Recumbency | - | +++ | ++ | +++ | - |
| Dullness | ++ | +++ | ++ | ++ | +++ |
| Rigidity | - | - | - | - | - |
| Tremors | - | - | - | - | - |
| Hyperactive | - | - | - | - | - |

a) in days, for both incubation and disease course

b) death by euthanasia

c) Grading marks, -, not observed; +, ++, +++, mild, moderate, marked clinical signs; ND, not determined

d) paresis of hind limbs

e) rigidity of extensor muscles

Table 8. Distribution of street rabies virus antigen as compared to CVS rabies virus antigen in the CNS of intranasally infected rabid skunks (for neurotransmitter studies).

| Brain region/nucleus | Skunk street ^a | CVS ^b |
|-----------------------------------|---------------------------|------------------|
| Gracile n. ^c | + | + |
| Cuneate n. | + | + |
| Inferior olivary n. | + | + |
| Accessory cuneate n. | + | + |
| Spinal n. of V | +++ | + |
| Dorsal motor n. of X | ++ | + |
| Hypoglossal n. | + | - |
| Abducens n. | + | + |
| Cochlear n. | + | ++ |
| Facial n. | ++ | - |
| Motor n. of V | ++ | + |
| Vestibular n. | ++ | ++ |
| Trochlear n. | + | - |
| Superior olivary n. | + | + |
| Nuclei pontis | - | - |
| Purkinje cells | - | ++ |
| Deep cerebellar n. | + | ++ |
| Red n. | ++ | - |
| Substantia nigra | ++ | ++ |
| Medullary raphe nuclei | ++ | + |
| Pontine raphe nuclei | ++ | + |
| Midbrain raphe nuclei | +++ | ++ |
| Dorsal raphe nucleus ^d | ++ | + |
| Medullary reticular nuclei | + | ++ |
| Pontine reticular nuclei | + | ++ |
| Mesencephalic n. | + | + |
| Interpeduncular n. | + | + |
| Ventral periaqueductal grey | +++ | ++ |
| Dorsal periaqueductal grey | + | + |
| Oculomotor n. | + | + |

a) average of 5 animals

b) average of 5 animals

c) n. - nucleus

d) Rostral margins, measured at the level of the oculomotor nucleus.

Grading: -, no immunoperoxidase staining present; +, ++, +++, +++++, mild, moderate, marked, and very marked immunoperoxidase staining.

serotonin and met-enkephalin in paraffin embedded brain tissue. The distribution of rabies antigen within the brains of rabid skunks shown in Table 8, was the same as previously reported in Chapter 4. There was decreased intensity of immunoperoxidase staining observed in both the ventral periaqueductal grey matter and raphe regions through out the medulla, pons and midbrain (as compared to Chapter 4), for both skunk street and CVS rabies viruses. This was most likely due to the less than optimum staining conditions.

The distribution of street rabies virus or CVS rabies virus did not coincide with the pattern of distribution for the two neurotransmitters evaluated in this experiment.

Detection of serotonin

The intensity and distribution of serotonin immunoperoxidase staining is also summarized in Table 9. The DAB reaction product was visualized as homogenous staining which filled the cell body or fibre. It varied in colour from light brown (mild staining) to a very dark brown or black color (very marked staining). The majority of serotonin positive cell bodies were located in the medullary, pontine, and midbrain raphe nuclei as well as the ventral periaqueductal grey matter. The midbrain raphe nuclei were located in an area which extended from the rostral pons to approximately the level of oculomotor nucleus in the cranial aspect of the midbrain. The dorsal raphe nucleus of the

midbrain was considered to be located in the ventral periaqueductal grey matter and dorsal midbrain raphe, with the rostral limit to be in the region located between the oculomotor nuclei. Only a few serotonin-containing cells were located in what was considered to be the rostral limit of the dorsal raphe nucleus. Immunoreactive fibres were also visualized in several brain stem nuclei. This distribution was similar to that seen in the CNS of rats (Steinbusch et al., 1978) and human foeti (Takahashi et al., 1986).

Figure 5 shows the very marked immunoperoxidase staining that occurred in cells and fibres at the level of the caudal ventral periaqueductal grey matter. Cells which were located in the pontine raphe, midbrain raphe and ventral periaqueductal grey matter had marked to very marked staining. This is consistent with the observation that these regions contain the nuclei which produce the majority of serotonin found in the CNS (Steinbusch, 1978).

There was no difference in the intensity or distribution of serotonin immunoperoxidase staining for skunk street virus or CVS rabies viruses as compared to control skunks. (Table 9) (Figure 6).

Table 9. Serotonin immunoreactivity in the CNS of control skunks and experimentally infected rabid skunks for street rabies virus as compared to CVS rabies virus.

| Brain region | Control ^a | | Street virus ^b | | CVS virus ^c | |
|---------------------------------|----------------------|-------|---------------------------|-------|------------------------|-------|
| | fibres | cells | fibres | cells | fibres | cells |
| Gracile n. ^d | + | - | + | - | + | - |
| Cuneate n. | + | - | + | - | + | - |
| Inferior olivary n. | + | - | ++ | - | + | - |
| Accessory cuneate n. | - | - | - | - | - | - |
| Spinal n. of V | + | - | ++ | - | ++ | - |
| Dorsal motor n. of X | ++ | - | | | | |
| Hypoglossal n. | + | - | | | | |
| Abducens n. | - | - | - | - | - | - |
| Cochlear n. | - | - | - | - | - | - |
| Facial n. | ++ | - | ++ | - | ++ | - |
| Motor n. of V | ++ | - | ++ | - | ++ | - |
| Vestibular ni ^e | - | - | - | - | - | - |
| Trochlear n. | - | - | - | - | - | - |
| Superior olivary n. | + | - | + | - | + | - |
| Nuclei pontis | + | - | + | - | + | - |
| Purkinje cells | - | - | - | - | - | - |
| Deep cerebellar ni. | - | - | - | - | - | - |
| Red n. | + | - | + | - | + | - |
| Substantia nigra | +++ | - | ++ | - | ++ | - |
| Medullary raphe ni. | + | +++ | ++ | +++ | + | ++ |
| Pontine raphe ni. | ++ | ++++ | +++ | +++ | ++ | +++ |
| Midbrain raphe ni. | +++ | ++++ | +++ | +++ | +++ | ++++ |
| Dorsal raphe ni. ^f | +++ | +/- | ++ | +/- | ++ | +/- |
| Medullary retic. ni. | ++ | - | + | - | + | - |
| Pontine retic. ni. | ++ | - | + | - | + | - |
| Mesencephalic n. | + | - | + | - | + | - |
| Interpeduncular n. | +++ | - | +++ | - | +++ | - |
| Ventral peri. grey ^g | +++ | ++++ | +++ | ++++ | +++ | +++ |
| Dorsal peri. grey | + | - | + | - | + | - |
| Oculomotor n. | - | - | - | - | - | - |

Average of a) 2 animals, b) 4 animals, c) 4 animals.

d) n. - nucleus

e) ni. - nuclei

f) Rostral margin, measured at the level of the oculomotor nucleus.

g) periaqueductal grey matter

Grading: -, no immunoperoxidase staining present; +, ++,

Figure 5. Immunoperoxidase staining of serotonin containing cells of the ventral periaqueductal grey matter of an uninfected control skunk. (MAG 400X)

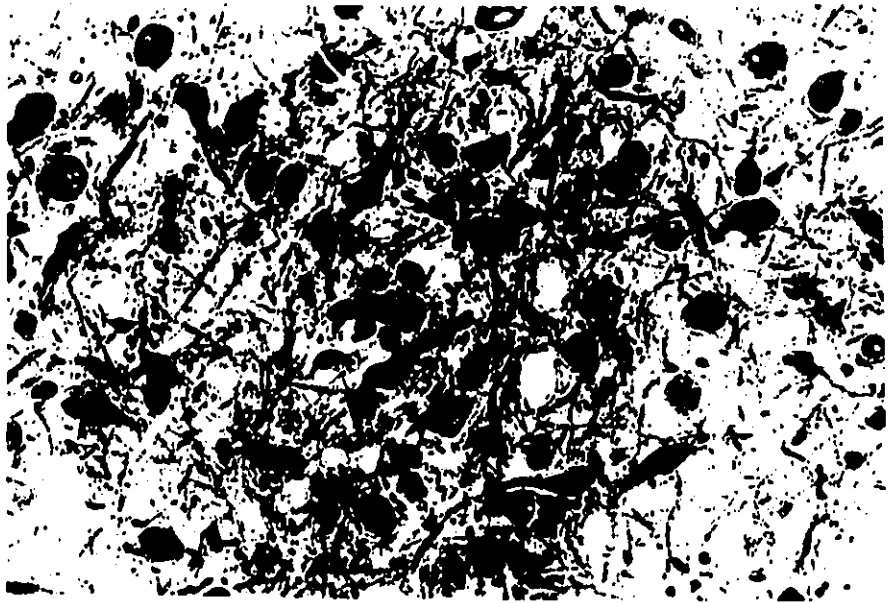


Figure 6. Immunoperoxidase staining of serotonin containing cells of the ventral periaqueductal grey matter and dorsal raphe nucleus of uninfected (A) as compared to street rabies virus infected (B) as compared to CVS rabies virus infected (C) skunks. (MAG A,B,C, 40X)



A



B



C

Met-enkephalin

Met-enkephalin immunoreactivity was observed only in fibres. Immunoperoxidase staining was present in most of the brain regions studied in the control skunks as shown in Table 10. The most intense reaction was found in the region of the globus pallidus where very marked staining was observed (Figure 7A). The reaction product was present as a homogenous light brown (mild staining) to a black colour (marked staining) which appeared to fill nerve fibres. The distribution of this neurotransmitter in control skunks was consistent with that reported in the literature for monkeys (Haber and Elde, 1982). A reduction in immunoperoxidase staining was observed in brains infected with both skunk street and CVS rabies viruses as compared to the intensity of immunoperoxidase staining seen in uninfected control skunks (Figure 7). It is unclear from these data if there was a significant difference in the intensity of staining between the skunk street virus and CVS rabies infected brains.

Table 10. Met-enkephalin immunoreactivity in the CNS of experimentally infected rabid skunks for street rabies virus as compared to CVS rabies virus.

| Brain region | Control ^a | Street virus ^b | CVS virus ^c |
|------------------------------|----------------------|---------------------------|------------------------|
| Gracile n. ^d | ++ ^c | - | - |
| Cuneate n. | ++ | - | - |
| Inferior olivary n. | ++ | + | + |
| Accessory cuneate n. | ++ | - | - |
| Spinal nucleus of V | ++ | + | - |
| Dorsal motor n. of X | ++ | ++ | + |
| Hypoglossal n. | ++ | + | + |
| Abducens n. | - | - | - |
| Cochlear n. | - | - | - |
| Facial n. | + | + | - |
| Motor n. of V | - | - | - |
| Vestibular nuclei | - | - | - |
| Trochlear n. | - | - | - |
| Superior olivary n. | + | + | + |
| Pontine nuclei | - | - | - |
| Purkinje cells | - | - | - |
| Granular layer, cerebellum | - | - | - |
| Deep cerebellar nuclei | ++ | - | - |
| Red n. | + | - | - |
| Substantia nigra | ++ | + | + |
| Medullary raphe nuclei | + | + | + |
| Pontine raphe nuclei | + | - | - |
| Midbrain raphe nuclei | ++ | + | - |
| Dorsal raphe n. ^f | - | - | - |
| Medullary reticular nuclei | ++ | + | + |
| Pontine reticular nuclei | ++ | + | - |
| Mesencephalic n. | + | - | - |
| Interpeduncular n. | +++ | + | - |
| Ventral peri. grey | ++ | + | + |
| Dorsal peri. grey | ++ | + | + |
| Oculomotor n. | + | - | - |
| Pineal body | + | - | - |
| Pituitary | +++ | | |
| Habenular nuclei | - | - | - |
| Hippocampal formation | - | - | - |
| Dentate gyrus | - | - | - |
| Thalamus | + | - | - |
| Nucleus subthalamicus | + | - | - |
| Hypothalamus | + | + | + |
| Amygdaloid nucleus | - | - | - |
| Caudate nucleus | + | - | - |
| Septal nuclei | - | - | - |
| Cortex | + | - | - |
| Globus pallidus | ++++ | +++ | ++ |

Table 10. continued

Average of a) 2 skunks, b) 4 skunks, c) 4 skunks

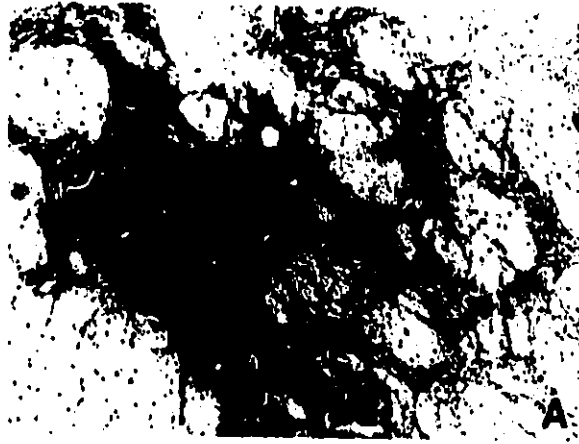
d) n. - nucleus

e) immunoperoxidase staining present in fibres only

f) Rostral margins, measured between the oculomotor nucleus.

Grading: -, no immunoperoxidase staining present; +, ++, +++, +++++, mild, moderate, marked, and very marked immunoperoxidase staining.

Figure 7. Immunoperoxidase staining of met-enkephalin containing fibres and cells of the globus pallidus in uninfected (A), street rabies virus infected (B) and CVS rabies virus infected (C) skunks. (MAG A,B,C, 400X)



Discussion

These results provide further information about two current theories on rabies pathogenesis; namely that cells producing specific neurotransmitters are trophic for rabies virus as first claimed by Johnson in 1965, and that inhibitory neurotransmitter level(s) might be reduced in rabies infected neurons (Tsiang, 1988).

These data do not support the theory that rabies virus selectively accumulates in neurons containing the inhibitory neurotransmitters serotonin or met-enkephalin. The overall pattern of distribution of either virus in the CNS was not restricted to cells in which serotonin was detected. Both street rabies virus and CVS rabies virus were present in areas of serotonin immunoreactivity as well as in regions where serotonin was not detected. For example, street virus was present in greater amounts than CVS virus in serotonergic fibres of the facial nucleus, however CVS virus was present in fibres of the interpeduncular nucleus, a structure with marked immunoreactivity for serotonin. The ventral periaqueductal grey, a region shown to be associated with aggressive behavior contained slightly more street virus than CVS rabies virus. However, differential staining was not observed in this region in the previous experiment. The different tissue fixatives used in these two experiments may account for this observed difference. The overall intensity of rabies immunoperoxidase staining was reduced in this experiment as compared to the

previous one described in Chapter 4. A reduction in background staining might account for the observation of this small difference seen in the intensity of rabies immunoperoxidase staining. It was more difficult to interpret the pattern of met-enkephalin immunoperoxidase staining because immunoreactive fibres were diffusely distributed throughout the brain. Since cell bodies did not show any met-enkephalin immunoperoxidase staining, it was difficult to determine the origin of positive staining fibres. Rabies virus did not appear to be preferentially associated with regions of met-enkephalin immunoperoxidase staining. Even regions of very marked immunoperoxidase staining (globus pallidus and pituitary) showed little or no accumulation of rabies virus antigen.

The intensity of serotonin immunoperoxidase staining of rabies infected serotonergic CNS neurons was similar for both rabid (street or CVS) and uninfected control skunks. This finding does not however, rule out the possibility that other interruptions in serotonin metabolism might occur as a result of rabies virus infection of the CNS. The immunoperoxidase technique would only have detected changes in the steady state levels of serotonin and not other metabolic malfunctions such as receptor interference, failure of serotonin release or dysfunction of effector cells. It is also possible that this method was not sensitive enough to demonstrate a clinically significant change in the level of serotonin within cells.

The midbrain raphe nuclei at the level of the oculomotor nucleus contained less serotonergic cell bodies than was at first anticipated from studies in Chapters 3 and 4. In this area only a few serotonergic neurons and fibres were observed. It is most likely that this region contains the caudal aspect of the Edinger-Westphal nucleus which is found in association with the oculomotor nucleus. It is unclear if or how the apparent accumulation of rabies virus in a nucleus which processes visual information, might be associated with the onset of biting behavior. The reason for the different accumulation of CVS versus street rabies virus in this region is unknown at this time.

The intensity of immunoperoxidase staining of met-enkephalin was decreased in rabid (street and CVS viruses) versus control animals. This was observed throughout the CNS and could not be attributed to the accumulation of virus in any particular region. The data from this experiment were not sufficient to provide an explanation for this observation. Since all tissues were subjected to identical fixation procedures and each staining batch included tissues from each of the three experimental groups, this observation is not due to variations in tissue staining or handling. Although rabies virus has not been shown to decrease protein synthesis in the host cell (Madore and England, 1977), it is possible that the post translational processing step required in met-enkephalin synthesis (Stewart, 1989), is disrupted by rabies virus

infection of neurons. The effect of this apparent decrease in met-enkephalin levels is difficult to evaluate because although met-enkephalin is considered to be an inhibitory neurotransmitter, its exact effect on behavior has not been clearly defined (Frederickson and Geary, 1982).

CHAPTER 6**General discussion and conclusions.**

Despite the rapid accumulation of data on the biology of rabies virus in the past twenty years (Tsiang, 1988), the pathogenesis of clinical signs which occur in rabid animals has yet to be elucidated. Recently developed diagnostic techniques including immunoperoxidase staining, as described in this thesis, have revealed new information about the dynamics of rabies virus within the central nervous system of rabid animals. With the use of immunoperoxidase staining, these experiments showed that the distribution of rabies virus in the brains of experimentally infected rabid skunks and the clinical presentation of the disease could be influenced by the strain of rabies virus used for infection. It was also shown for skunk street rabies virus only, that the pattern of virus distribution in the CNS was the same regardless of whether the skunks were inoculated intramuscularly or intranasally with rabies virus. The different patterns of distribution between these two rabies viruses may be one possible explanation for the two clinical forms of the disease (dumb and furious) described in this study. However, additional experimentation is required to further confirm and substantiate this possibility.

These data also showed that the pathogenesis of rabies induced by CVS rabies virus (a fixed virus) is not entirely

similar to that of street rabies virus which causes naturally occurring infection. This is the first study in which an animal model that supports enzootic rabies in nature has been used to study the pathogenesis of behavior changes of street rabies virus infection. As a result, the viral events occurring during early stage street rabies virus CNS infection have been described and can now be compared to that which has been documented in previously established animal models of rabies.

The basis for rabies virus tropism in the CNS is unknown at present. Although particular regions of the viral genome have been identified as determinants of virulence (Tuffereau et al., 1989; Lafay et al., 1991), there is no conclusive evidence as to whether rabies virus tropism in the CNS is host or virus mediated. Recent reports have implicated neurotransmitter substances or their neuronal cell receptors as potential trophic factors which might influence the infection of the CNS and the replication of rabies virus within neurons (Lentz 1982; Gosztonyi, 1984; Gourmelon et al., 1991). If rabies virus does accumulate in association with a particular neurotransmitter, this phenomenon could explain both the pattern of accumulation of rabies virus in the CNS as well as behavioral changes seen in rabid animals. In this study however, neither street rabies virus or CVS rabies virus accumulated preferentially in neurons or fibres containing serotonin or met-enkephalin. However, while infection of

neurons with rabies virus did not appear to reduce the intensity of immunoperoxidase staining for serotonin, the intensity of immunoperoxidase staining for met-enkephalin was reduced in both skunk street rabies virus and CVS rabies virus infected skunks. The following discussion will address these findings with respect to the current understanding of rabies virus infection of the CNS and the potential interaction of rabies virus with the inhibitory neurotransmitters serotonin and met-enkephalin.

Since immunoperoxidase staining technology (Hsu, 1981) has been a relatively recent development, there are only a few published studies of rabies pathogenesis which might be compared to this research (Feiden, 1985; Feiden et al., 1987, Bourgon, 1987; Jackson and Reimer, 1989; Jackson, 1991). Generally, our findings for dumb rabies (CVS rabies virus induced) are consistent with those in the studies reported by Feiden, (1985), Feiden et. al., (1987), Jackson and Reimer, (1989), and Jackson, (1991); however, their lack of anatomical description reduces the comparisons of rabies virus antigen accumulation to brain regions rather than to individual nuclei. Our observations on furious rabies (skunk street rabies virus infection) differ from these studies (listed above) in the clinical signs described and in the amount of virus detected in the cerebellum. In the study of Feiden et al., (1987), the data were presented as a summary of many

different wild species such that individual differences between species could not be identified. In addition it was not possible to determine from the experimental material if all animals had been infected by the same rabies virus strain. Jackson and Reimer (1989) and Jackson (1991), used the mouse as an animal model for the study of CVS rabies virus distribution by immunoperoxidase staining; however, this species is not suitable for the study of enzootic rabies and in addition, only specific regions of the brain were evaluated. Therefore, the observations presented in this thesis are the first and only available documentation of the pattern of distribution of rabies virus during early stage street rabies virus infection at the present time.

More information is available on the pathogenesis of CVS rabies virus in laboratory animal models. The detailed nature of the observations in this thesis shows that previously unobserved differences in the accumulation of CVS rabies virus versus skunk street rabies virus do occur. These results indicate that the pathogenesis of street and fixed rabies viruses may be quite different. It is tempting to speculate that a greater understanding of this difference might provide further insight into the mechanism of the behavior changes which occur in rabies disease.

The basis for the differing patterns of distribution of skunk street rabies virus and CVS rabies virus in the CNS is still completely unknown. The observation in Chapter 3 that

street virus inoculated either IM or IN leads to the same pattern of virus distribution during the early stages of clinical rabies gives some support for the notion that a rabies virus mediated tropism for particular CNS neurons exists. It is possible that virus either fails to infect certain neurons of the CNS or it infects but does not replicate equally well in all of the structures that it infects. Further studies are required to determine whether either of these possibilities account for virus accumulation in specific CNS regions. Since intramuscular CVS rabies virus infection failed to cause rabies, no data were available for comparison with regard to this route of inoculation.

These data have not provided a basis for an explanation of why two skunks in the experiments described in Chapter 3, failed to show biting behavior despite having a CNS distribution of rabies virus similar to other animals in the experiment. This discrepancy was not observed in the following inoculation experiments. Although these two animals failed to bite, they did not exhibit clinical signs that were exactly the same as those shown by skunks inoculated with CVS virus showing dumb rabies. Although they were dull and unresponsive to external stimuli, they did not exhibit the muscle rigidity and recumbency characteristic of CVS rabies virus infected skunks which were described in Chapters 4 and 5. Perhaps the two street rabies virus infected skunks which failed to show biting behavior, had sufficient virus induced

dysfunction in other brain regions to reduce these skunk's biting capabilities.

The consequences of impaired serotonergic inhibitory control in humans have been listed as; insomnia, hyperemotionality, hyperirritability, neophobia and anxiety, increased susceptibility to convulsive seizures, pain sensitivity, avoidance learning, irritative aggression, sexual behavior (priapism), behavioral disinhibition, violent, pathological and suicidal aggression (Valzelli, 1984). Animal models of hyposerotonergic neurotransmission are difficult to evaluate in terms of emotionality changes however; some similarities between human and animal conditions of hyposerotonism have been observed. Lesioning of the raphe nuclei (a major producer of serotonin in the CNS) in the rat, has been shown to lead to an increase in aggressive behaviors as measured by muricide (Pucilowski and Kostowski, 1981; Grant et al., 1973), as well as increases in defensive aggression and hyperemotionality (Yamamoto and Ueki, 1977). Pucilowski and Kostowski (1981) demonstrated that stimulation of the dorsal raphe nuclei reduced the mouse killing behavior of rats.

In a comprehensive discussion on the role of serotonin neurons in human and animal behavior, Soubrie (1986), presented the viewpoint that reduced serotonin transmission generally leads to a release of behaviors usually suppressed

under normal circumstances. He cited behaviors characteristic of decreased serotonin neurotransmission as being; increased locomotor activity in rodents following median raphe lesions and increased muricide by rats.

The similarity of the clinical signs of rabies to the behavioral activities associated with reduced serotonin neurotransmission, particularly in skunks showing the furious form of the disease warrants further investigation. The predominant clinical signs of early stage furious rabies were found to be hyperactivity and an increase in aggressive/biting behavior. In contrast, skunks exhibiting the dumb form of rabies became dull and unresponsive to external stimuli. From these data it was not clear if the skunks may have passed quickly through a stage of excitement, if the dullness resulted from additional CNS impairments which masked an excitable stage, or if the pathogenesis of CVS rabies virus is sufficiently different that it cannot be compared to street rabies virus. However, in the case of street rabies virus the possibility that a decrease in serotonin neurotransmission might account for the clinical signs observed, cannot yet be ruled out based on the results of this study. Other metabolic dysfunctions such as receptor interference, failure of serotonin release or decreased reactivity of effector cells are a few mechanisms which could lead to clinical hyposerotonism and a general increase in aggressive behavior.

Both street and CVS rabies viruses were present in the

midbrain raphe neurons in marked to very marked amounts. Only in the rostral limit of the dorsal raphe nucleus (at the level of the oculomotor nucleus) was a difference in rabies antigen accumulation observed. This region however was shown by immunoperoxidase staining to contain less serotonin than was at first anticipated. As a result of this finding, it is likely that this area includes part of the Edinger-Westphal nucleus, a nucleus associated with vision (contraction of the pupil and ciliary muscles). The significance of the differences in accumulation of street rabies virus as compared to CVS rabies virus in this region with respect to the occurrence of biting behavior remains in question. Additional experimentation is required to determine if there are significant changes in the levels of serotonin in this region during rabies infection.

Another region which merits further investigation is the ventral periaqueductal grey matter. This region has also been shown to play a major role in the modulation of aggressive behavior (Bandler, 1988; Seigel and Brutus, 1990). Seigel and Brutus, 1990 stated that biting behaviors could be elicited by stimulation of sites in the dorsal periaqueductal grey matter as well as of sites located lateral to the aqueduct in the medial aspect of the periaqueductal grey matter. In addition, Bandler (1988), described sites within the periaqueductal grey matter that upon stimulation elicited various components of aggressive behavior such as vocalization, head position, body

stance and movement. In Chapter 5, it was noted that more street rabies virus antigen was observed in the ventral periaqueductal grey matter than for CVS rabies virus. This might have occurred as a result of a different antigen detection method (different fixation) as compared to Chapters 3 and 4, however, this aspect deserves further study considering the importance ascribed to the periaqueductal grey matter with respect to aggressive behavior.

The very marked accumulation of CVS rabies virus in the Purkinje cells of the cerebellum also provided an opportunity to evaluate the effects of very marked accumulation of rabies virus antigens on neuronal physiology. Immunoperoxidase staining of the inhibitory neurotransmitter GABA (produced in large quantity by the Purkinje cells of the cerebellum) (Otterson and Storm, 1984; Alger 1985) was chosen as a measure of this effect. Challenge Virus Standard rabies virus infected skunks appeared to have reduced immunoperoxidase staining for GABA in Purkinje cell bodies and processes, however, the quality of the staining was unacceptable for inclusion of this data in the final results. Stained sections showed tremendous variation in the quality and intensity of staining within and between sections. During the course of this experiment, it was not possible to determine if this finding was due to staining technique or if in fact, it was a true indication of GABA distribution within this region. Further investigation of the effect of rabies virus infection

on GABA levels within Purkinje cells is warranted to determine if the heavy accumulation of CVS rabies virus antigen in these cells interferes with neurotransmitter production.

This is the first study in which a decrease in met-enkephalin immunoreactivity in rabies infected skunk brain has been demonstrated. Several explanations for this observation arise from these initial findings. A direct effect of rabies virus replication on the neuron might cause an interference with the production of the protein pre-proenkephalin or its post-translational products proenkephalin or met-enkephalin. Since the antibody was specific for the end product only, it was not possible in this experiment to determine at which stage of catabolism the interruption might have occurred.

Alternatively, the apparent reduction in steady state levels of met-enkephalin may have occurred indirectly due to viral induced dysfunction of functionally interconnected neurons. For example, it has been shown that denervation of the adrenal causes a decrease in the levels of pre-proenkephalin mRNA (Franklin et al., 1991). This observation may be interpreted as an indication that there is an apparent need for transynaptic impulse activity in order to maintain the steady state levels of PPenk and met-enkephalin end products. If this is the case, reduced levels of neurotransmission in rabies virus infected brain (as has been proposed by Tsiang (1988)), might lead to insufficient

neuronal stimulation for the purposes of sustained neurotransmitter production. On the other hand, this effect might occur at the cell membrane receptor. Morris and Hunt (1991) showed that the application of a D2 antagonist in the striatum caused a decrease in the levels of proenkephalin mRNA. If rabies virus binds to specific neuronal receptors, (a theory which has been incompletely proven at present), it is possible that the reduction in CNS levels of met-enkephalin could be mediated by rabies-host cell receptor binding by an as yet unknown mechanism.

The complex nature of neurotransmitter interactions in the CNS has made the interpretation of experimental data extremely difficult since multiple effects often result from the manipulation of even one neurotransmitter. Increased levels of met-enkephalin, for example, have been shown to suppress the excitatory amino acids, aspartate and glutamate (Bishop 1991). Interactions have also been documented with other neurotransmitters, such as dopamine and others. A reduction in the level of met-enkephalin throughout the CNS as demonstrated in this study, could be expected to lead to a disinhibition of excitatory neurotransmitters, the results of which might be consistent with the clinical signs of furious rabies as described in this study.

Interactions between met-enkephalin and serotonin producing neurons have also been identified. Wang et al., (1991), showed with double immunostaining that the rat dorsal

raphe nucleus contains many met-enkephalin terminals and that some of these make synaptic contacts with soma and dendrites of the 5 HT DRN neurons. Currently, the origin of these fibres is unknown. It has also been shown in the cat that met-enkephalin terminals are present in some of the raphe nuclei (Wang et al., 1991). This finding might provide an explanation of how, despite the observation that serotonin levels seemed unaffected in the rabies infected brain, a dysfunction in serotonin neurotransmission may occur. If met-enkephalin is required to facilitate serotonin neurotransmission, reduced serotonin activity may be mediated through this effect. The similarity between syndromes of hyposerotonism and clinical rabies should not be left uninvestigated.

In conclusion, the principal accomplishments of the study are as follows:

1. This is the first report to describe the distribution of skunk street rabies virus in the CNS of experimentally infected rabid skunks in early stage rabies. This description is provided for both the intramuscular and intranasal route of inoculation.
2. This is the first report which describes the distribution of CVS rabies virus in the CNS of experimentally infected

rabid skunks in early stage rabies.

3. Differences are described for the pattern of distribution of skunk street rabies virus as compared to CVS rabies virus in the brains of experimentally infected rabid skunks during early stage rabies.

3. The dorsal raphe nucleus was identified as a potential mediator of biting behavior in skunks experimentally infected with skunk street rabies virus.

4. The intensity of immunoperoxidase staining for serotonin in the CNS of skunks does not decrease as a result of experimental rabies infection with either skunk street rabies virus or CVS rabies virus.

5. The intensity of immunoperoxidase staining for met-enkephalin was reduced in the CNS of skunks infected with either skunk street rabies virus or CVS rabies virus.

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