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Early events in rabies virus infection of the central nervous system in skunks (*Mephitis mephitis*)

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Abstract Twenty-four striped skunks were inoculated intramuscularly (long digital extensor muscle of right pelvic limb) with street rabies virus. Groups of two clinically normal skunks were killed at various times after inoculation; skunks that developed rabies were killed in early stages of the clinical signs. Four clinically normal skunks (numbered 1–4) had slight infection in lumbar spinal ganglia, spinal cord and brain. These four skunks were used for detailed immunohistochemical (rabies antigen) studies that included examination of sections from every segment of the spinal cord, most of the spinal ganglia from the 2nd cervical to the 2nd coccygeal (sections at 25-μm intervals of lumbar, sacral and coccygeal ganglia) and brain (sections at 50-μm intervals). In skunks 1–4, there was increasing distribution of antigen-containing neurons that was not correlated with the time elapsed since inoculation. In three skunks (nos. 1, 2 and 3), antigen-containing neurons were predominantly in caudal regions of the spinal cord, caudal right lumbar and sacral spinal ganglia and certain nuclei/regions of the brain (medial reticular formation, right interpositus and lateral vestibular nuclei, left red nucleus, left motor cortex, and left reticular nucleus of the thalamus). Skunk 4 had more extensive infection than skunks 1–3, but the previous pattern was still evident. The results are consistent with viral entrance into the lumbar spinal cord, initial replication mainly at the L2 and L3 levels, local spread in the cord by propriospinal neurons and early transit to the brain via long ascending and descending fiber tracts (bypassing the grey matter of the rostral spinal cord). These mechanisms could provide for early and rapid dissemination in the brain before a significant immune response develops and could induce behavioral changes before the animal is incapacitated by extensive spinal cord infection. Based on the distribution of antigen-containing neurons, the tracts considered most likely to serve as viral transitways from spinal cord to brain include: rubrospinal, corticospinal, spinothalamic, spino-olivary, vestibulospinal and/or spinovestibular, reticulospinal and/or spinoreticular, cerebellospinal and/or spinocerebellar, and dorsal column pathways.

Key words Rabies · Skunk · Immunohistochemistry · Viral transit in fiber tracts · Pathogenesis

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Introduction

The pathogenesis of rabies generally involves bite-inflicted deposition of virus-laden saliva into tissues (skin, muscle) of a susceptible animal, migration of virus up peripheral nerves to the central nervous system (CNS), spread throughout the CNS, and centrifugal spread in peripheral nerves to infect some non-nervous tissues ([4–6, 15, 16, 20, 42, 43, 48, 49], for reviews see [3, 13, 14, 31, 51]). Amplification of infection in the nervous system occurs through cycles of viral replication (mainly in neurons) and cell to cell transfer of progeny virions. The electron microscopic features of viral replication in neurons [15, 38, 42, 43, 46], and a mechanism of transneuronal transfer of infection [15, 32] have been described. There is substantial evidence that transport of the infectious agent in both the peripheral nervous system (PNS) and CNS occurs in axons and the mechanism of transport is axoplasmic flow ([11, 12, 26, 54], for review see [55]). Several studies suggest that infection in the CNS after entry into the spinal cord (following inoculation of one of the legs) generally spreads in a spatially integrated fashion from the entry site to most areas of the spinal cord and brain [4, 29, 30, 34, 35, 43, 49]. Most of these studies utilized laboratory animals infected with fixed rabies virus strains, and characterized spread of infection to general regions (medulla oblongata, pons, etc.) rather than to specific nuclei of the CNS, providing very few data on specific pathways and specific brain nuclei/regions affected early in the disease.

We have studied early events in CNS infection in a host species (striped skunks, *Mephitis mephitis*) infected with street virus. Part of the difficulty in studying the sequence of changes in street virus infections in host species is the variability of the incubation period, even in animals given the same dose of virus [17]. In the present study, we infected striped skunks with moderate doses of street rabies virus and killed infected animals at various times after inoculation. Four skunks in preclinical stages of the disease had very slight to moderate spread of infection in the CNS and were used for detailed immunohistochemical studies of the distribution of antigen-containing neurons in spinal ganglia, spinal cord and brain.

Materials and methods

Experimental animals

Striped skunks *(M. mephitis)* were obtained from a supplier (Ruby's Fur Farm) in New Sharon, Iowa. They were kept in stainless steel cages and given food and water ad libitum. The experiment followed the guidelines of the Canadian Council on Animal Care.

Virus

A 20% suspension w/v of submandibular salivary glands of naturally infected skunks (in Ontario) was prepared by homogenization in diluent [0.01 *M* phosphate buffer, pH 7.4, containing 10% fetal bovine serum (FBS), 1000 IU penicillin and 2 mg streptomycin sulfate/ml] and centrifugation at 3000 rpm for 20 min. The supernatant was stored at –70° C until used. Following preliminary titration, inoculum was prepared by dilution of the supernatant to 10^{-2} dilution of original infected salivary glands using the above phosphate buffer containing 2% FBS. The inoculum contained $10^{5.6}$ mouse intracerebral median lethal doses ($MICLD_{50}$)/ml as determined by intracerebral inoculation of weanling white Swiss mice immediately after inoculation of skunks.

Experimental procedure

Each of 24 skunks was anesthetized by intramuscular (IM) administration of 2 mg acepromazine maleate (Ayerst Laboratories, Montreal, Quebec) and 160 mg ketamine hydrochloride (Rogar STB, London, Ontario) followed by 5 mg of Rompun (Miles Laboratories, Rexdale, Ontario). Using aseptic surgical procedures, each skunk was IM inoculated (long digital extensor muscle of the right pelvic limb) with 0.1 ml of inoculum. Two skunks did not receive any inoculum and were used as controls.

Two clinically normal skunks (one male, one female) were killed on each of days 1, 2, 4, 7, 10, 14 and 21; 3 were killed on day 42. Seven other skunks developed clinical rabies on days 17 (n = 2), 19, 22, 24, 25 or 28 and were killed in early stages of the disease. All skunks were necropsied. Tissues collected at necropsy included brain, spinal cord, nearly all spinal ganglia from the second cervical to the second coccygeal roots and selected visceral tissues. They were fixed in phosphate-buffered formalin pH 5.3, dehydrated and embedded in paraffin. Abbreviations for regions of the spinal cord and associated ganglia are: cervical (C), thoracic (T), lumbar (L), coccygeal (Cg).

Immunohistochemistry and distribution of antigen-containing neurons

Preliminary examination of sections of selected tissues stained by an avidin-biotin complex (ABC) method [6] revealed four skunks in early (nonclinical) stages of CNS infection. These four skunks (the subject of this report) were used for detailed immunohistochemical studies that included every segment of spinal cord and nearly all the spinal ganglia from C2 to Cg2, and the entire brain.

Three (5 μm) sections were cut at 25-μm intervals of spinal ganglia from C2 to T13, and from every segment of the spinal cord. (In addition, sections at 25-μm intervals were cut throughout the entire block C5 from skunks 1 and 2). The remaining ganglia (T14, T15, L1-5, S1 and S2, Cg 1 and 2) were serially sectioned. Sections were taken at 25-μm intervals and stained by the ABC method [6]. To compare the extent of infection among dorsal root ganglia of individual skunks, estimates of the percent of examined neurons that contained antigen were made as follows in most af-

Table 1 Skunk 1: distribution of rabies antigen in spinal cord and dorsal root ganglia (*C* cervical, *T* thoracic, *L* lumbar, *S* sacral, *Cg* coccygeal, *NE* not examined, *IZ* intermediate zone, *VH* ventral horn, *DH* dorsal horn)

Segment of spinal cord	Neurons containing antigen		
	Left spinal ganglion[a]	Spinal cord[b]	Right spinal ganglion[a]
C1	NE	–	NE
C2	NE	–	–
C3	–	–	–
C4	–	–	–
C5	–	–	–
C6	–	–	–
C7	–	–	–
C8	–	–	–
T1	–	–	–
T2	–	–	–
T3	–	–	–
T4	–	–	–
T5	–	–	–
T6	–	–	–
T7	NE	–	–
T8	–	± IZ (1 neuron)	–
T9	–	–	–
T10	NE	± IZ (1 neuron)	–
T11	–	–	NE
T12	–	–	–
T13	–	–	–
T14	–	± IZ,VH	–
T15	–	± IZ,VH	–
L1	–	± IZ,VH	–
L2	–	+ IZ,VH,±DH	< 1%
L3	–	+ IZ,VH,±DH	3.6%
L4	–	+ IZ,VH	5.9%
L5	< 1% (1 neuron)	± VH	4.9%
S1	–	± VH	1%
S2	–	–	< 1%
Cg1	–	T[c]	–

[a] – indicates no antigen-containing neurons detected; percentage, indicates estimate of percent of examined neurons that contained antigen (calculated from counts of neuronal profiles as described in text)

[b] The extent of positive neurons is indicated as follows: none (–); 1–5 positive neurons (±); and approximately one-third (+), two-thirds (++), and all (+++) of the neurons examined in the designated area

[c] Spinal cord terminated in previous segment

Table 2 Skunk 2: distribution of rabies antigen in spinal cord and dorsal root ganglia (abbreviations as for Table 1)

Segment of spinal cord	Neurons containing antigen		
	Left spinal ganglion[a]	Spinal cord[b]	Right spinal ganglion[a]
C1	NE	–	NE
C2	–	–	–
C3	–	–	–
C4	–	–	–
C5	–	–	–
C6	–	–	–
C7	–	–	–
C8	–	± IZ (1 neuron)	–
T1	–	–	–
T2	–	–	–
T3	–	–	NE
T4	–	–	–
T5	–	± IZ (2 neurons)	–
T6	–	–	NE
T7	NE	–	–
T8	–	–	–
T9	–	–	–
T10	NE	± IZ (1 neuron)	–
T11	–	± IZ (1 neuron)	–
T12	NE	± IZ,VH	–
T13	NE	± IZ,VH	–
T14	–	± IZ,VH	–
T15	–	± IZ, VH	–
L1	–	+ IZ,VH	–
L2	–	++ IZ,VH,±DH	< 1%
L3	< 1% (2 neurons)	++ IZ,VH,+DH	< 1%
L4	< 1%	± IZ,VH,DH	10.3%
L5	< 1%	± IZ,VH,DH	8.4%
S1	< 1%	± IZ	5.2%
S2	< 1%	–	< 1%
Cg1	< 1%	–	< 1%
Cg2	–	T[c]	1%

[a] – indicates no antigen-containing neurons detected; percentage, indicates estimate of percent of examined neurons that contained antigen
[b] The extent of positive neurons is indicated as in Table 1
[c] Spinal cord terminated in previous segment

Table 3 Skunk 3: distribution of rabies antigen-containing neurons in spinal cord and dorsal root ganglia (abbreviations as for Table 1)

Segment of spinal cord	Neurons containing antigen		
	Left spinal ganglion[a]	Spinal cord[b]	Right spinal ganglion[a]
C1	NE	–	NE
C2	NE	–	–
C3	NE	± VH (1 neuron)	–
C4	–	–	–
C5	–	± VH (1 neuron)	–
C6	–	± IZ (2 neurons)	–
C7	–	± IZ (1 neuron)	–
C8	–	± IZ,VH	–
T1	–	± IZ,VH	–
T2	–	± IZ,VH	–
T3	–	± IZ,VH	–
T4	–	± IZ,VH	–
T5	–	± IZ	–
T6	–	± IZ,VH	–
T7	–	± VH	< 1%
T8	–	± IZ,VH	–
T9	–	± IZ,VH	< 1%
T10	–	± IZ,VH	–
T11	< 1%	± IZ,VH	< 1%
T12	–	± IZ,VH	< 1%
T13	NE	± IZ,VH	< 1%
T14	< 1%	± IZ,VH,DH	< 1%
T15	< 1%	+ IZ,VH, ± DH	< 1%
L1	< 1%	+ IZ,VH,±DH	< 1%
L2	< 1%	++ IZ,VH,±DH	NE
L3	2.7%	++ IZ,VH,±DH	7.1%
L4	2.6%	± IZ,VH,DH	16.5%
L5	2.0%	± IZ,VH,DH	11.4%
S1	< 1%	–	6.7%
S2	< 1%	–	1.3%
Cg1	< 1%	T[c]	2.9%
Cg2	–		2.0%

[a] – indicates no antigen-containing neurons detected; percentage, indicates estimate of percent of examined neurons that contained antigen
[b] The extent of positive neurons is indicated as in Table 1
[c] Spinal cord terminated in previous segment

fected ganglia. In sections at 100-µm intervals, profiles of neurons that contained stained antigen were counted (designated positive neurons) regardless of whether or not nuclei were visible in the section. Similar neuronal profiles containing Nissl granules, but not antigen, were counted and designated as negative neurons. Trials in which serial sections were stained and examined indicated that only neurons were counted and that the same neurons were not counted in sections at 100-µm intervals. These counts (positive/positive and negative) for all sections of a given ganglion were used to estimate the percentage of neurons that contained antigen. Preliminary studies indicated that ganglia with an average of four or less positive neurons per section generally had less than 1% positive neurons. Total neurons in such ganglia were not counted and the results were recorded as < 1%. The various ganglia were assumed to be similar in structure. There is no evidence that early stage rabies is characterized by changes in number, size or shape of neurons, features that could result in bias in estimates of ratios [18]. The same investigator (K. C.) made all counts.

The 1st cervical segment of the spinal cord and the coronal slices of the entire brain were serially sectioned at 5 µm and sections at 50-µm intervals were used for ABC staining for rabies antigen. Computer drawings (CorelDRAW, Corel Corp., Ottawa, Ontario) were prepared from selected Nissl-stained coronal sections of normal skunk brain. These drawings were used to prepare summary diagrams, each diagram containing the total positive neurons in all the sections examined in a specific caudal-rostral region. The same set of 11 caudal-rostral regions was used to record positive neurons in all four skunks. The approximate locations of the positive neurons were indicated by symbols placed in affected brain nuclei/regions. (Right and left are as they appear in the diagrams.) This procedure has the advantage of emphasizing the consistencies among sections – a feature that is especially important when individual sections contain only a few positive neurons.

Results

Of the 24 skunks, 7 developed clinical rabies after incubation periods of 17 (n = 2), 19, 22, 24, 25 or 28 days. Positive neurons (neuronal perikarya contained stained antigen) were widespread in the brain, spinal cord and spinal ganglia. The lesions in skunks with clinical rabies have been reported previously [15, 52] and are not described here.

Of the 17 clinically normal skunks, no antigen was detected in 13; 4 had slight to moderate numbers of positive neurons in spinal cord, spinal ganglia and brain. The 4 skunks were numbered 1–4 in order of increasing distribution of antigen. The skunks and days of euthanasia are as follows: skunk 1, day 10; skunk 2, day 21; skunk 3, day 42; skunk 4, day 14 (Tables 1–4; Fig. 1–4). The lesions in skunks 1–3 are described first, since dissemination of infection was much less than in skunk 4.

The distributions of positive neurons in the spinal cords, spinal ganglia and brains of skunks 1, 2 and 3 were remarkably similar, differing moderately in extent of infection and slightly in regions involved. In the spinal cord, the most severely affected segments were L2 and L3 (Ta-

Table 4 Skunk 4: distribution of rabies antigen in spinal cord and dorsal root ganglia (abbreviations as for Table 1)

Segmant of spinal cord	Neurons containing antigen		
	Left spinal ganglion[a]	Spinal cord[b]	Right spinal ganglion[a]
C1	NE	± IZ	NE
C2	–	± IZ	–
C3	–	± IZ	–
C4	–	± IZ	–
C5	–	± IZ,VH	–
C6	–	± IZ,VH	–
C7	–	± IZ,VH	–
C8	–	± IZ,+VH	–
T1	–	± IZ,VH,DH	–
T2	–	± IZ,VH,DH	–
T3	–	± IZ,VH,DH	–
T4	–	± IZ,VH,DH	–
T5	–	± IZ, VH	–
T6	–	± IZ,VH,DH	–
T7	–	± IZ,VH,DH	–
T8	NE	+ IZ,±VH,DH	–
T9	–	+ IZ,±VH	< 1%
T10	–	++ IZ,+VH,±DH	< 1%
T11	–	++ IZ,VH,±DH	< 1%
T12	NE	++ IZ,+VH,±DH	< 1%
T13	< 1%	++ IZ,VH,DH	< 1%
T14	NE	++ IZ,VH,+DH	4.0%
T15	7.7%	++ IZ,+++VH,+DH	5.8%
L1	5.5%	++ IZ,VH,+DH	8.9%
L2	3.4%	+++ IZ,VH,DH	NE
L3	3.8%	++ IZ,VH,++DH	18.9%
L4	10.1%	+++ IZ,VH,++DH	36.6%
L5	8.4%	+++ IZ,VH,++DH	29.2%
S1	9.8%	± IZ	26.8%
S2	6.0%	T[c]	17.6%
Cg1	NE		13.9%
Cg2	7.8%		14.3%

[a] – indicates no antigen-containing neurons detected; percentage, indicates estimate of percent of examined neurons that contained antigen

[b] The extent of positive neurons is indicated as in Table 1

[c] Spinal cord terminated in previous segment

Figs. 1–4 Summary diagrams depicting positive neurons in various caudal-rostral regions of the brain. To compare the distribution of positive neurons in the skunks, the same set of 11 caudal-rostral regions was used for recording results. Those regions not depicted for some skunks did not contain antigen. The regions are as follows: *Region 1* Caudal poles of gracilis and medial cuneate nuclei to immediately caudal to the hypoglossal nucleus and dorsal motor nucleus of cranial nerve (CN) X. *Region 2* Caudal poles of hypoglossal nucleus and dorsal motor nucleus of CN X to the obex. *Region 3* Obex to rostral poles of hypoglossal and dorsal motor nucleus of CN X. *Region 4* Immediately rostral to hypoglossal nucleus and dorsal motor nucleus of CN X to immediately caudal to motor nucleus of CN 7. *Region 5* From caudal to rostral poles of motor nucleus of CN 7. *Region 6* Caudal superior olive to caudal motor nucleus of CN 5 and rostral extremity of nucleus interpositis. *Region 7* Caudal pole of motor nucleus of CN 5 to immediately rostral to this nucleus. *Region 8* Caudal midbrain (decussation of the trochlear nerve) to caudal extremity of trochlear nucleus. *Region 9* Caudal region of trochlear/oculomotor complex to rostral extremity of the red nucleus. *Region 10* Posterior commissure to immediately rostral to the infundibulum. *Region 11* Optic chiasm to genu of the corpus callosum ▶

Fig. 1 A–C Skunk 1. Figures and corresponding regions: A,4; B,6; C, 9

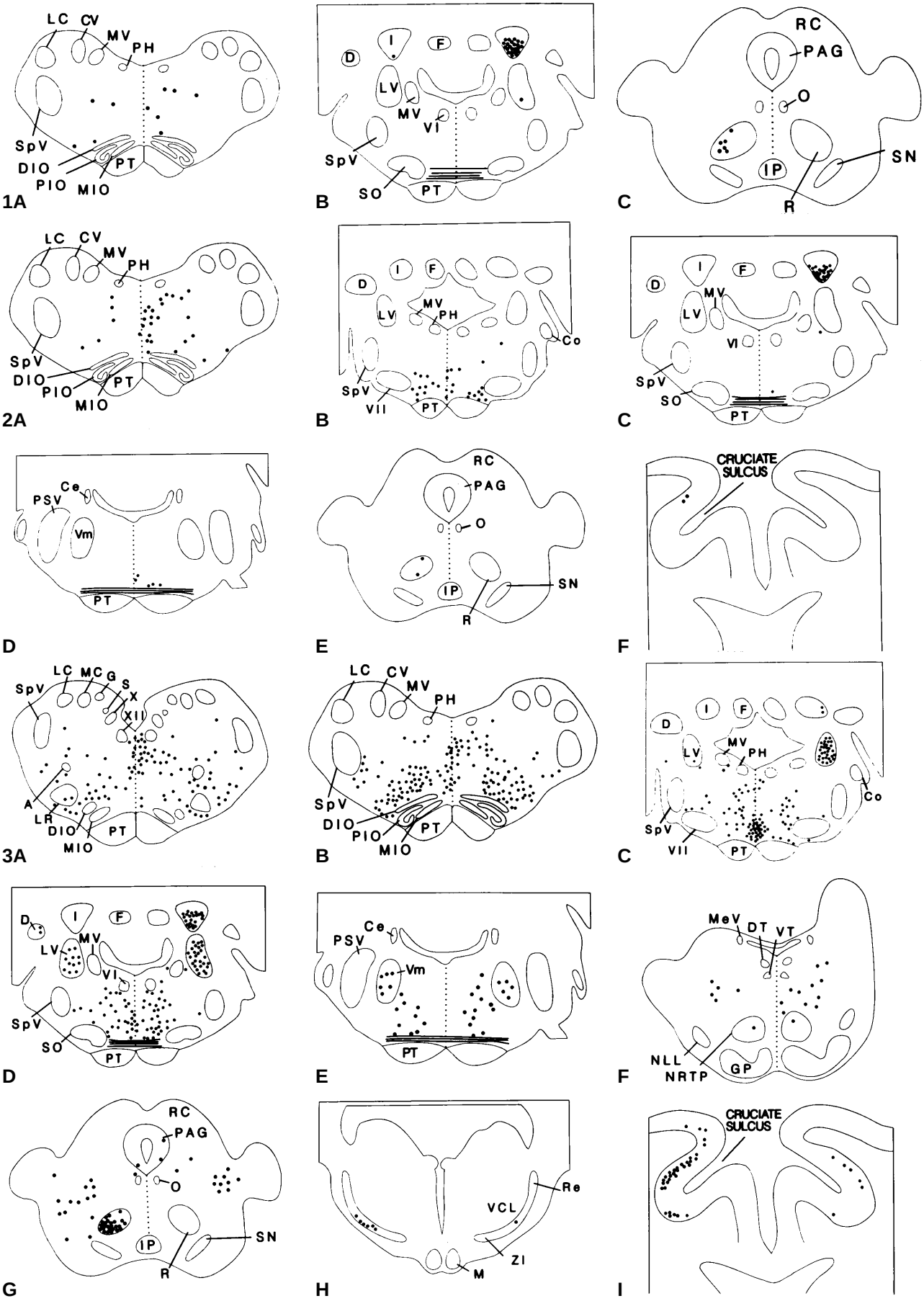
Fig. 2 A–F Skunk 2. Figures and corresponding regions: A,4; B,5; C,6; D,7; E,9; F,11

Fig. 3 A–I Skunk 3. Figures and corresponding regions: A,3; B,4; C,5; D,6; E,7; F,8; G,9; H,10; I,11

Fig. 4 A–K Skunk 4. Figures and corresponding regions: A,1; B,2; C,3; D,4; E,5; F,6; G,7; H,8; I,9; J,10; K,11

For Figs. 1–4 the following abrreviations and symbols have been used. Where appropriate, the terminology recommended by Nomina Anatomica Veterinaria, 4th ed is in parenthesis:

A ambiguus nucleus, *Ce* nucleus ceruleus, *Co* ventral cochlear nucleus, *CV* caudal vestibular nucleus, *D* dentate nucleus, *DIO* dorsal accessory inferior olivary nucleus (dorsal accessory olivary nucleus), *DT* dorsal tegmental nucleus, *F* fastigial nucleus, *G* gracilis nucleus, *GP* griseum pontis, *I* interpositus nucleus, *IP* interpeduncular nucleus, *LC* lateral cuneate nucleus, *LR* lateral reticular nucleus, *LV* lateral vestibular nucleus, *M* mamillary body, *MC* medial cuneate nucleus, *MeV* mesencephatic nucleus of the fifth cranial nerve, *MIO* medial accessory inferior olivary nucleus (medial accessory olivary nucleus), *MV* medial vestibular nucleus, *NLL* nucleus of the lateral lemniscus, NRTP nucleus reticularis tegmenti pontis, *O* oculomotor complex, *PAG* periaqueductal gray matter, *PH* nucleus prepositus hypoglossi, *PIO* principal inferior olivary nucleus (olivary nucleus). *PSV* pontine sensory nucleus of CN V, *PT* pyramidal tract, *R* red nucleus, *RC* rostral colliculus, *Re* reticular nucleus of the thalamus, *S* nucleus of the solitary tract, *SN* substantia nigra, *SO* superior olive (dorsal nucleus of the trapezoid body), *SpV* nucleus of spinal tract of CN V, *VII* motor nucleus of CN VII, *VM* motor nucleus of CN V, *VCL* ventral caudal lateral nucleus of the thalamus, *VT* ventral tegmental nucleus, *X* dorsal motor nucleus of CN X, *XII* hypoglossal nucleus, *ZI* zona incerta, *VI* abducens nucleus. The following symbols for positive neurons are indicated as follows: • (small dot) = 1, ● (large dot) = 5; ∗ = 10; ⁍ = 50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Figs. 1–3

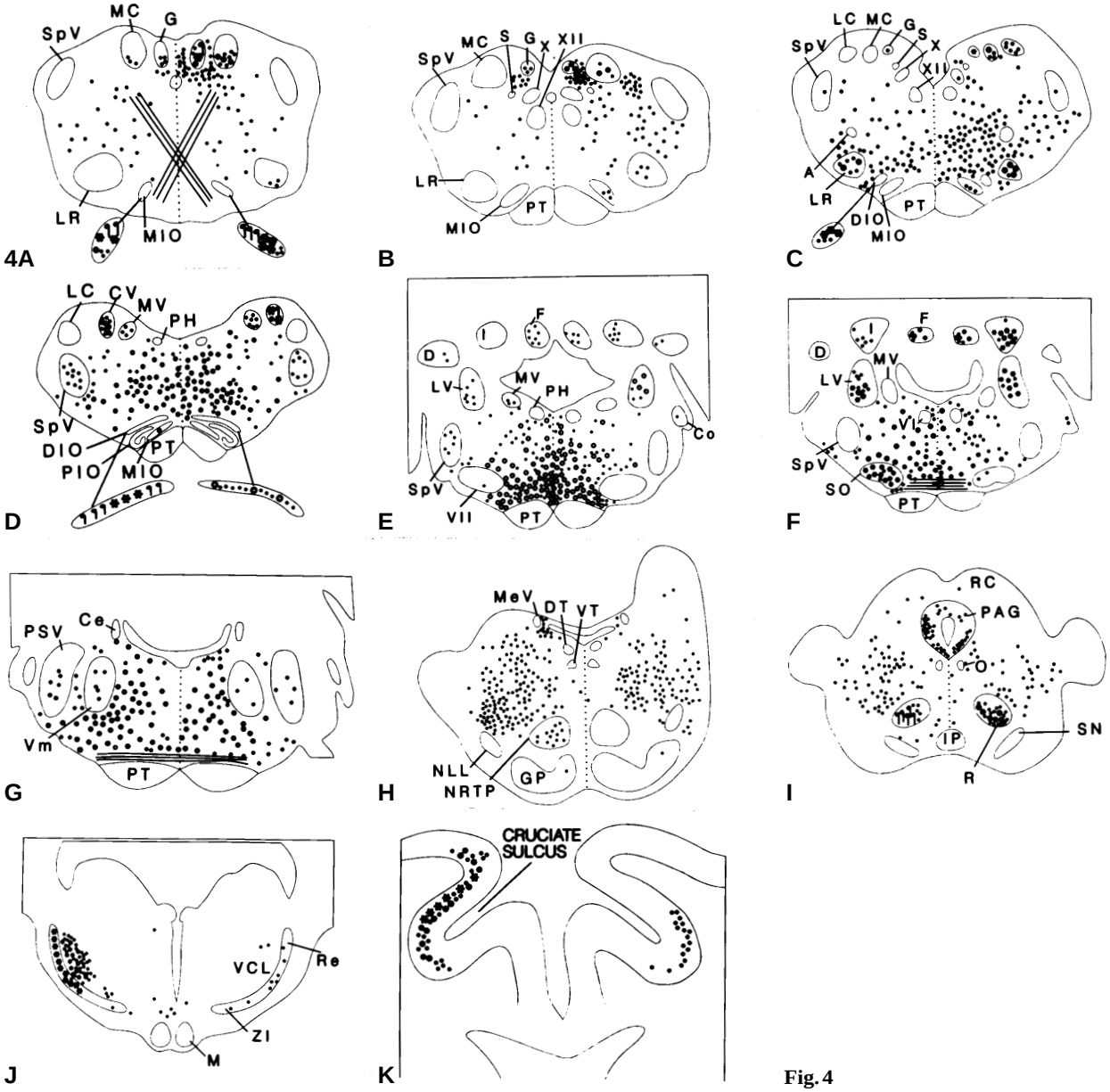

Fig. 4

bles 1–3) in which most of the positive neurons were unilateral, and were most numerous in the lateral column of ventral motor neurons (Fig. 5). Stained antigen occurred as fine to moderately sized brown-black granules in neuronal perikarya and processes. There was only slight infection of the dorsal horn. In rostral spinal cord, the sparse positive neurons were mainly in the intermediate zone (IZ) (Tables 1–3) and were more frequent on the right than on the left side. In the caudal thoracic and rostral lumbar cord, all three skunks had positive neurons in the nucleus dorsalis; skunk 3 had a few in the intermediolateral column. Spinal ganglia on the right side were always affected more severely than those on the left side with right lumbar ganglia 4 and 5 containing the greatest proportion of neurons that were positive (Tables 1–3). (Gross examination of the spinal cord and ganglia of normal skunks revealed that after entrance of the roots through the dura mater at L4 and 5, the bundles of fibers progress rostrally, under the dura mater, for approximately two segments before entering the parenchyma of the spinal cord. For embedding, segments were always taken at the point of root entrance through the dura mater. Thus, the most severe spinal cord lesions at L2 and L3 correspond to the most severely affected spinal ganglia at L4 and L5). In spinal ganglia, antigen occurred mainly in medium-sized to large neurons with relative sparing of small neurons. Frequently, granules of antigen were most prominent immediately beneath the perikaryal plasma membrane and, in favorable sections, antigen was detected in processes near perikarya. In skunk 4, although all segments of the spinal cord and most of the ganglia caudal to T8 were affected, the regional differences in severity of lesions were similar to skunks 1–3 (Table 4).

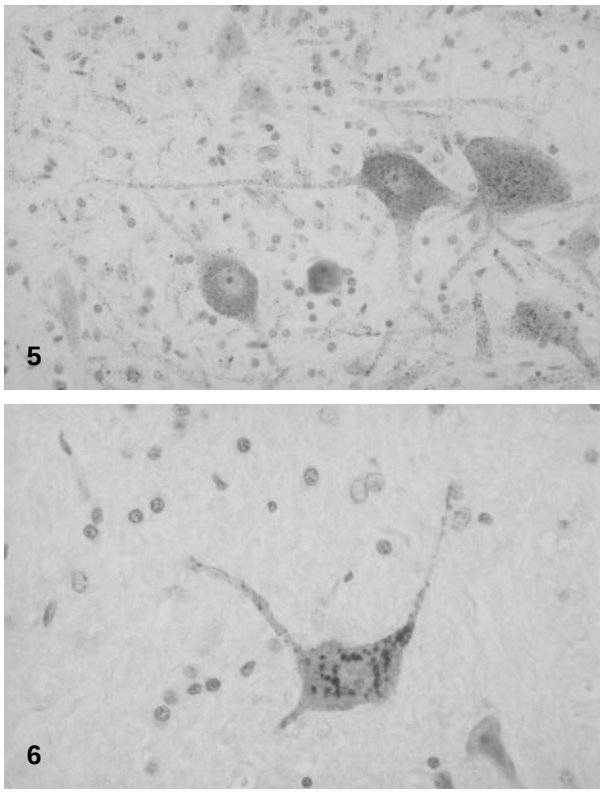

Fig. 5 Skunk 1. Rabies antigen in neuronal perikarya and dendrites of the lateral column of ventral motor neurons in the 3rd lumbar segment of the spinal cord. × 292

Fig. 6 Skunk 2. Rabies antigen in solitary neuron in this field of the medullary reticular formation. × 292

The principal brain nuclei/regions affected in skunks 1–3 included: the medial reticular formation (bilateral), left red nucleus, right nucleus interpositus, right lateral vestibular nucleus, left reticular nucleus of the thalamus and left motor cortex (Figs. 1–3). In the reticular formation, motor cortex, lateral vestibular and red nuclei, mainly large neurons were affected (Fig. 6). The number and extent of positive neurons increased in skunks 1–3. Positive neurons in the right interpositus nucleus were mainly rostroventral (Figs. 1–3). In the left red nucleus of skunk 3, positive neurons were mainly in the ventrolateral portion, a pattern that could not be detected in skunks 1 and 2 because of the small number of positive neurons. The medial side and tip of the postcruciate gyrus of the cerebrum comprising part of the motor cortex [45] was affected in both skunks 2 and 3 (Figs. 2, 3). Positive neurons were only seen on the left side of skunk 2, mainly on the left side of skunk 3, and included Betz cells and other large neurons in a band parallel to the innermost regions of the cortex (Fig. 3).

Although positive neurons in the brain of skunk 4 were more extensive than in skunks 1–3, most of the patterns in the first three were still evident (Fig. 4). There was more extensive involvement of the reticular formation extending from the caudal medulla oblongata to rostral midbrain (with regional concentrations immediately adjacent to the right gracilis and cuneate nuclei, ventral raphe area of the caudal pons, lateral regions in the rostral pons and central tegmental fields of the caudal midbrain). The distribution (rostroventral) of positive neurons in the right nucleus interpositus was similar to previous skunks; however, other deep cerebellar nuclei now contained scattered positive neurons. Positive neurons were very numerous in the left red nucleus, and were predominantly in the ventrolateral portion of the nucleus. The reticular nucleus (mainly left) of the thalamus was more severely affected than in skunk 3, and positive neurons were now in the ventral caudal lateral nucleus. Additional regions of the brain stem that now contained positive neurons included: gracilis and medial cuneate nuclei (mainly right side), right and left lateral reticular nuclei, right and left caudal vestibular nuclei, nuclei of the spinal tract of the trigeminal nerve, pontine sensory and motor nuclei of the trigeminal nerve, the periaqueductal gray matter, the medial accessory and dorsal accessory inferior olive (in which nearly all neurons contained antigen) and the superior olive. A very few Purkinje cells contained antigen. Except for the inferior olive, these regions/nuclei contained only a few scattered positive neurons. The cerebral cortex (motor area) adjacent to the cruciate sulcus (from level of optic chiasm to the genu of the corpus callosum) contained more positive neurons than this region in skunks 2 and 3. In addition to Betz cells and other large neurons, there were a few foci (deep in the cortex) of small neurons that contained antigen. Except for three positive neurons in the lateral part of the left postcruciate gyrus, other regions of the cerebral cortex and the hippocampus were negative.

Discussion

The increase in number of positive neurons in skunks 1–4 and the similarities in regions affected are interpreted as evidence of increasing progression of the infection, and of consistency in mechanisms of viral spread. The distribution of positive neurons especially in skunks 1–3 (mainly caudal spinal cord and several brain regions corresponding to origins or terminations of descending or ascending tracts) suggests spread of infection (to the brain) via long fiber tracts, thus bypassing the gray matter of the rostral spinal cord.

Our results suggest some general mechanisms involved in various features of the clinical disease. These features include development of behavioral changes before the animal becomes recumbent, avoidance of an effective immune response, dissemination of virus by non-cerebrospinal fluid (CSF) routes, development of hyperresponsiveness to external stimuli and retention of alertness during early clinical signs. Early migration of virus to the brain via long fiber tracts would be consistent with early induction of behavioral changes before spinal cord infection is severe enough to cause recumbency. Similarly, this rapid dissemination of CNS infection could provide for development of widespread brain infection before there is time for a significant immune response. It is likely that viral

dissemination in the CNS is consistently rapid, regardless of the length of the incubation period. In most natural infections in man, there is no detectable immune response until infection is well advanced (after onset of clinical signs) [24]. A similar delay in detectable antibodies occurs in experimental animals infected with low to moderate doses of virus [7, 17, 36]. Our recent unpublished studies indicate that skunks given the same dose of virus as that used in this study do not have detectable serum antibodies until onset of clinical signs. There is evidence that experimental slowly progressing CNS infections of mutant rabies virsues can be terminated by the immune response [19, 21, 59], suggesting that the rate of progression of CNS infection is important in producing the usual fatal outcome of rabies.

In the early stages (skunks 1 and 2), positive neurons in the brain were remote from the ependyma of the ventricular system suggesting that, at least in early stages of infection, viral movement in CSF is not a factor in viral spread. This is supported by studies in hamsters in which there was no infection of ependymal or meningeal cells [43], and evidence that axoplasmic flow serves as a vehicle of viral transport [11, 12, 25]; however, transit in CSF may be a factor in mice inoculated intracerebrally [33]. The predominance of positive neurons in the medial reticular formation indicates infection of neurons in the nuclei raphe pallidus and magnus, regions involved in modulation of sensory impulses arriving in the spinal cord [9, 47]. This and the paucity of involvement of small primary sensory neurons and the infrequent involvement of the dorsal horn (retention of pain pathways) may contribute to the hyperresponsiveness to external stimuli that occurs in early stages of the clinical disease [52].

The restriction of infection to the motor cortex with sparing of the remaining cortex and hippocampus early in viral dissemination, as detected in our skunks, probably accounts for the alertness in animals and periods of lucidity and comprehension in early stage human rabies [24].

Several studies have demonstrated that rabies virus/antigen first appears in regions of the CNS having direct neural connections to the inoculation site [5, 29, 30, 33, 34, 49, 50, 56, 57]; spread of the CVS strain of virus within the brain after intranasal [2] or intraocular [22] inoculation has been described. However, there are very few published data on details of spread from an entry site in the spinal cord to the brain (i.e., on transitways to and sequence of infection of particular nuclei of the CNS). One study of experimental CVS virus infection in mice suggested that spinocerebellar tracts were involved [33]. The distribution and extent of infected neurons in our skunks indicate that spread occurred both locally in the spinal cord and via several long fiber tracts. The relatively high concentrations of positive neurons in the spinal cord at L2 and L3 and in right dorsal root ganglia at L4, L5 and S1 suggests viral entrance into the cord via dorsal roots of these ganglia and/or corresponding ventral roots. Spread of infection in the cord beyond L2 and L3 probably occurred via propriospinal neurons. Most of these neurons are in the IZ (the region affected most frequently in the rostral spinal cord).

Except for the reticular formation, infection in various brain nuclei was to a large extent unilateral, frequently corresponding to the sites of origin or termination of ascending and/or descending fiber tracts. This early “unilateral” distribution suggests that viral transit to the CNS occurred soon after infection of the spinal cord (before extensive spread to the side contralateral to the inoculation site). Viral transport to target nuclei via indirect route(s) is considered unlikely since many susceptible brain regions receiving fibers from the reticular formation (affected early in course of viral spread) were not affected in our skunks. Also since the reticular formation was affected bilaterally, any subsequent transfer of infection could be expected to be bilateral. A similar argument holds for collaterals of descending axons. Assuming viral transit by the most direct routes, we consider the following tracts the most likely viral pathways: rubrospinal, corticospinal, spinothalamic, spino-olivary, vestibulospinal and/or spinovestibular, spinocerebellar and/or cerebellospinal, reticulospinal and/or spinoreticular, and dorsal column fibers to the nucleus gracilis and descending fibers from the same.

The early occurrence of positive neurons in the red nucleus and motor cortex, mainly contralateral to the inoculation site, is consistent with retrograde viral transit in rubrospinal and corticospinal tracts, respectively. The rubrospinal tract is “almost purely crossed” [10]. In the cat and monkey, rubrolumbar neurons are located mainly in the ventrolateral part of the nucleus [10, 28], thus corresponding to the ventrolateral distribution of positive neurons in our skunks. Similarly the distribution of positive neurons (mainly medial contralateral motor cortex) in our skunks and known termination patterns of corticospinal fibers [1] suggests viral migration from the lumbar spinal cord via corticospinal tracts. The occurrence of positive neurons in the lateral vestibular nucleus is consistent with viral migration in either or both spinovestibular and lateral vestibulospinal tracts. Recent studies suggest that fibers from the medial and caudal vestibular nuclei also project to lumbar levels and that there are spinal projections to all four major vestibular nuclei [41]. Possibly these connections account for the scattered positive neurons detected in the caudal and medial vestibular nuclei in skunk 4.

Although there are species differences in details of the destinations of spinothalamic fibers [44, 58], in most species there is a substantial contribution to the lateral thalamus – especially the ventral caudal lateral (posterolateral) nucleus [23, 47, 58]. The location of positive neurons in the contralateral ventroposterior complex of skunk 4 is consistent with anterograde transport via the spinothalamic tract.

In the rat, direct spino-olivary fibers terminate in the medial accessory inferior olive and the dorsal accessory inferior olive [53], regions that were severely affected in skunk 4, while other parts of the inferior olive were negative. Our findings are consistent with transit of rabies virus from the spinal cord via this route.

The interpositus nucleus, ipsilateral to the inoculation site, contained positive neurons in all skunks including

those least affected (skunks 1 and 2), suggesting early migration of virus from the lumbar cord. In a study of experimental CVS rabies virus infection of mice, the deep cerebellar nuclei were prominently involved on day 5 post infection (before antigen was detected in the cerebral cortex or hippocampus [33]. Some fibers in the dorsal and ventral spinocerebellar tracts project to the fastigial and interpositus nuclei in the cat, rat and rabbit [39, 40] and probably are the most likely routes for viral transit to the interpositus nucleus in our skunks. Cerebellospinal fibers [8] may also be transitways for virus.

Involvement of the nuclei gracilis and cuneatus suggest viral transit in the dorsal columns, and/or in descending fibers from non-core and adjacent regions of these nuclei.

Because of the early and fairly extensive involvement of the reticular formation in our skunks, this region is obviously an important site of rabies virus infection of the brain. Based on the termination patterns of reticulospinal and spinoreticular fibers in various species [10, 27, 37, 58], our findings of bilateral distribution of positive neurons favor viral spread via reticulospinal fibers (many from the medulla and pons terminate bilaterally), but the spinoreticular route cannot be ruled out.

In summary, this study demonstrates that several long descending and ascending fiber tracts serve as conduits for spread of infection from the spinal cord to the brain. This would require viral transit via retrograde and anterograde axoplasmic flow, respectively, and corresponding dendroaxonal/somatoaxonal and axodendritic/axosomatic transfer of infection. The occurrence of rabies viral antigen in several groups of neurons suggests that viral specificity for specific types of neurons is not an important factor in early stages of infection.

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