Key words: rabies virus/Sindbis virus/neuromuscular junction/virus attachment

Entry of Rabies Virus into the Peripheral Nerves of Mice

By HOPE D. WATSON, GREGORY H. TIGNOR* AND ABIGAIL L. SMITH

Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510, U.S.A.

(Accepted 27 May 1981)

SUMMARY

Young adult mice were inoculated in the hind limb with rabies virus or Sindbis virus. Rabies 1820B virus antigen was detected in leg sections by immunofluorescence at 1 h post-inoculation at sites comparable in form and distribution to cholinesterase-positive sites, which represent motor end-plates (MEPs). Sites which were rabies virus antigen-positive by immunofluorescence were also cholinesterase-positive on double-stained slides. Rabies CVS virus detected by autoradiography was similarly distributed at 6 h post-inoculation. Uptake of rabies virus at motor nerve endings was confirmed by the detection of rabies antigen by immunofluorescence in ventral horn cells in the spinal cord at 20 h post-inoculation before involvement of dorsal root ganglia. Rabies virus antigen could not be detected at MEPs if the virus had been inactivated by beta propiolactone or mixed with antibody prior to injection or if the sciatic nerve had been cut 7 days earlier; similarly treated groups of mice survived for the observation period of 6 weeks. Rabies virus antigen was found at MEPs in mice given antibody 24 h before virus injection, but virus antigen was not found in the spinal cord, and mice similarly treated survived. Sindbis virus strain Ar86, which like rabies virus is neurotropic in adult mice, was also found at MEPs and in peripheral nerves by autoradiography at 6 h post-inoculation. In contrast to results with rabies virus-infected mice, stimulation of the sciatic nerve for the first hour post-inoculation prevented mortality. Sindbis virus strain Ar339, which is not neurotropic in adult mice, could not be detected at MEPs by immunofluorescence or autoradiography and mice injected with virus survived. The results presented here suggest that rabies virus and perhaps other neurotropic viruses can use the motor axon terminal at the neuromuscular junction as a site of entry into the nervous system.

INTRODUCTION

Much experimental evidence shows that rabies virus reaches the central nervous system (CNS) via peripheral nerves and that haematogenous spread of virus to the CNS is rare or of little importance. The techniques used in these studies include cutting of nerves before or after inoculation of virus, tissue titration, electron microscopy and fluorescent antibody staining (Baer, 1975; Murphy, 1977). It is not known when the uptake into nerves occurs, whether it involves both sensory and motor nerve endings, and whether the uptake process is mediated by specific host cell receptors at the nerve endings.

Dean et al. (1963) found that three out of four rabies (CVS) virus-infected guinea-pigs in which the dorsal roots were sectioned died, suggesting that virus ascends ventral roots. On the other hand, in a separate experiment, they found that two out of three guinea-pigs whose ventral roots were removed also died of rabies, suggesting that virus may also traverse the...
dorsal roots. In addition, they concluded from results of other experiments that virus demonstrated at the site of injection at 48 h post-inoculation might be there as a result of centrifugal spread from the CNS.

Johnson (1965), using immunofluorescence, did not find rabies (CVS) antigen in extraneural tissue, at the inoculation site, or in peripheral nerves after injection of virus into the hind footpad of adult mice. He first detected antigen in lumbar dorsal root ganglia and neurons in the spinal cord 3 days post-inoculation.

Schneider (1969) reported that virus multiplied first in spinal ganglia after footpad inoculation of mice. Virus was found at the site of inoculation for the first 6 h post-inoculation. However, the virus reached the CNS by 72 h without spinal ganglia involvement after intravenous injection. Injection of normal mouse brain tissue ('stimulation') simultaneously with virus inoculation in the hind limb did not change the site of entry of virus into the CNS.

Murphy et al. (1973a) studied the pathogenesis in 4-day-old hamsters of three strains of rabies virus (CVS, a vampire bat isolate and an arctic fox isolate) and two rabies-related viruses. After intramuscular (i.m.) injection in the hind limb, they found virus antigen by immunofluorescence along the needle tract in extracellular spaces for 24 h but not in other places. The temporal sequence of rabies virus infection observed in their laboratory model led Murphy and his colleagues to suggest that infection originated in striated muscle followed by entry into the nervous system in muscle and tendon spindles (i.e. via the sensory nerves).

The terminal portion of the axon at neuromuscular junctions (NMJs) is another possible site of entry for viruses into the nervous system (Rapoport, 1976) because the perineural sheaths do not cover the portion of the nerve facing the synaptic cleft which is accessible to the extracellular space. Since rabies virus has been found in extracellular spaces soon after injection (Schneider, 1969; Murphy et al., 1973a), it seemed likely that virus could diffuse into the vicinity of NMJs. We carried out the present study to see if the axon terminal at the NMJ might be involved as an entry site for rabies virus very early in the infection process.

Our experiments were conducted in mice with two strains of rabies virus, 1820B and CVS; for comparison, similar experiments were also done with two strains of an alphavirus, Sindbis, one strain of which is neurotropic.

**METHODS**

*Viruses.* Rabies virus strain 1820B (Sodja et al., 1971; Sodja & Matouch, 1971) stock originated from a rodent in Czechoslovakia and was passed two or three times after receipt from Dr T. Wiktor, Wistar Institute, Philadelphia, Pa., U.S.A. This strain is antigenically indistinguishable from rabies CVS virus in both serological and vaccination-challenge tests (G. H. Tignor & R. E. Shope, unpublished data). The fact that the incubation period in intracerebrally inoculated infant mice does not change with passage level suggests that the virus is 'fixed'. The use of this strain which kills all inoculated mice in a short period of time provided a workable model system for this type of study. Because the 1820B strain has not been grown to high titre in our laboratory, radiolabelled CVS strain of rabies virus was employed for experiments using autoradiography.

Challenge virus standard (CVS) rabies virus, derived from the original Pasteur strain, has been passed more than 2000 times in rabbits and an unknown number of times in mice. A plaque-cloned strain (Buckley & Tignor, 1975) was labelled with 5'-[^3H]uridine (New England Nuclear), 10 to 50 mCi/ml, in CER cells and purified by equilibrium gradient centrifugation using sucrose. The specific activity [ct/min per focus-forming unit (f.f.u.)] of the labelled CVS virus was 0-09.

Two biotypes of Sindbis, an alphavirus, were chosen for controls and for comparison. The Ar86 strain which is neurotropic for adult mice (Weinbren et al., 1956) was included in the
study to determine if virus association with the NMJ was a property unique to rabies virus which, in contrast to Sindbis virus, does not cause a prolonged viraemia. The Ar339 strain (Taylor et al., 1955) which does not kill adult mice (Johnson et al., 1972) was used to determine if virus association with NMJs occurred in the absence of lethal neurotropic disease. Sindbis virus strain Ar86 had undergone 65 mouse brain passages and Sindbis virus strain Ar339 was at the 15th mouse brain passage level. They were labelled with 5'-\[^3\text{H}\]uridine in CER cells as described elsewhere (Smith & Tignor, 1980). The specific activity (ct/min/f.f.u.) of the labelled Sindbis Ar86 strain was 0.1, and the specific activity of the labelled Sindbis Ar339 strain was 0.4.

Experimental design. Three- to 5-week-old Swiss albino mice (Taconic Farms, Germantown, N.Y., U.S.A.) were inoculated in the left hind limb by i.m. or intraplantar (footpad) route, or both, with 0-05 ml suspension containing approx. 10\(^5\) adult mouse intracerebral LD\(_{50}\) of rabies 1820B virus or 10\(^6\) fluorescent focus units of the other viruses. Because i.m. inoculation left a needle track in the early hours after inoculation, fluorescence results along this track within 24 h of inoculation with virus were interpreted with caution. Mice injected with Sindbis virus strains were inoculated in the same sites. Animals infected with rabies 1820B virus were killed at intervals up to 96 h, whereas animals infected with the other viruses were killed at early times only. The injected leg between ankle and knee and the spinal cord with spinal nerve roots attached were removed and examined by immunofluorescence (rabies 1820B virus and both Sindbis strains) and autoradiography (labelled rabies CVS virus and labelled Sindbis Ar86 and Ar339 viruses). The contralateral leg was examined at 72, 96 and 120 h post-inoculation. Experiments were repeated three to five times and fluorescence observations were made on one or two animals in each experiment. The location of NMJs was determined using histochemical techniques on tissues from both normal and rabies virus-infected mice. Rabies 1820B virus was also inoculated into mice after: (i) the virus had been inactivated with beta-propiolactone (BPL, 0-01% pH 9, 1 h at 37 °C); (ii) the virus had been mixed in equal proportions with antibody; or (iii) the mice had been inoculated intraperitoneally 24 h earlier with antibody. Tissues were examined by immunofluorescence at 1 and 96 h post-inoculation. Other mice were observed for death for 6 weeks.

Histology. Excised tissues were fixed in Lillie’s formalin and embedded in Paraplast (Lancer, St. Louis, Mo., U.S.A.). Serial transverse sections, 8 μm thick were cut and stained using Bodian’s method or the haematoxylin and eosin procedure.

Histochemistry. The location of NMJs was determined using the method of Karnovsky & Roots (1964) to detect acetylcholinesterase which is concentrated at motor end-plates (Beckett & Bourne, 1957; Hallpike, 1976). Tissues were fixed in cold 3 % glutaraldehyde in phosphate buffer pH 6.5 for 10 min and quick frozen in a polyethylene glycol medium (O.C.T. compound, Ames Co., Elkhart, Ind., U.S.A.) in a dry ice–alcohol slurry. They were cut in serial 8 μm transverse sections (120 μm between sections) and incubated for 45 min at room temperature in the incubation mixture containing 5 mg acetylcholine iodide (Eastman Kodak) in 6.5 ml. Some slides were counterstained with eosin; other slides were counterstained by the fluorescent antibody technique.

The method of Graham & Karnovsky (1966) was used to detect horseradish peroxidase (HRP). Frozen sections from the leg were incubated for 8 min in a solution of 3,3-diaminobenzine in 0-05 m-tris–HCl buffer pH 7.6 containing 0-01 % H\(_2\)O\(_2\).

Immunofluorescence. Serial frozen transverse sections of hind limbs, cut 120 μm apart, were fixed in acetone for 10 min at room temperature or 16 h at −20 °C. Sections from rabies virus-infected mice were stained by the direct method using fluorescein-labelled equine or bovine anti-rabies globulin (1:40) (BBL). Those from Sindbis virus-infected mice were stained by the indirect method using ascitic fluid (1:40) from hyperimmunized mice and fluorescein-labelled goat anti-mouse globulin (1:100) (Antibodies Inc., Davis, Ca., U.S.A.).
Sections were counterstained with Schiff’s reagent (1:200) (Fisher Scientific, Pittsburgh, Pa., U.S.A.) for 10 min to eliminate glutaraldehyde-induced fluorescence. After brief washing in distilled water, they were mounted in glycerol jelly or buffered glycerine. Sections of the spinal cord were 80 to 100 µm apart; 120 to 180 sections of each spinal cord were examined. Tissues from mock-infected mice and from mice infected with a different virus served as controls. All sections stained with fluorescent antibody were examined with an Olympus BH-B microscope using dark-field illumination, halogen light, an FITC exciter filter, and GG9 and 530 barrier filters.

Autoradiography. Tissues were fixed, embedded and cut as described under histology. After removal of paraffin, sections were coated with autoradiographic nuclear track emulsion (NTB no. 2, Eastman Kodak), diluted 1:2 with distilled water and then kept for 14 days at 4 °C in a dry atmosphere. Autoradiograms were developed with Kodak D19 developer at 20 °C for 3 min, washed in distilled water, fixed in Kodak fixer, and counterstained with haematoxylin and eosin.

Nerve section. Mice were anaesthetized with methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, N.J., U.S.A.). A skin incision about 20 mm long was made on the outer aspect of the thigh near the upper femur. The sciatic nerve was severed after exposing it by separating the muscles with blunt dissection; the animals were allowed to recover. Based upon the observation of Lentz et al. (1977) that nerve terminals had degenerated and disappeared in a mouse hemidiaphragm denervated for 1 week, virus was injected into the footpad 7 days later. Leg muscles were examined by immunofluorescence at 1 h post-inoculation. Other mice were held for 6 weeks.

Electrical stimulation. Mice were anaesthetized with methoxyflurane and the left sciatic nerve exposed (as above). Bipolar electrodes connected to an electronic stimulator (C. H. Stoelting Co., Chicago, Ill., U.S.A.) were placed in contact with the nerve. Mice were injected in the footpad of the same limb with either virus or 250 units HRP (Sigma) and then stimulated for 1 h (2-min periods of stimulation with 1-min rests). Enough voltage (0.5 to 5 V) was used to elicit strong contractions of the leg muscles. Frequency was set at 10 pulses/s and duration at 1 ms. Mock stimulation consisted of anaesthetizing the mice and placing the electrodes in contact with the sciatic nerve without current. Mice were anaesthetized during the entire stimulation procedure. Leg muscles were examined by immunofluorescence or histochemistry at 0.5 and 1 h post-inoculation to determine if stimulation increased uptake of virus or HRP. Other mice were observed for mortality.

RESULTS

Location of motor end-plates (MEPs)

Cholinesterase-positive sites were located in a scattered pattern within the muscles in association with muscle cells (Fig. 1 a to c). These sites within the muscles were variable in appearance reflecting the heterogeneity of MEP structure (Beckett & Bourne, 1957; Gauthier, 1976). On tissues which were cut in cross-section, the MEPs were located on the periphery of the muscle cells and were crescent-shaped.

Rabies 1820B virus: immunofluorescence

Virus antigen was detected by immunofluorescence at 1 h post-inoculation in sections from mice infected with rabies 1820B virus in either the footpad or in both leg muscle and footpad in a distribution similar to that of cholinesterase-positive sites within the bodies of muscles. These fluorescent sites were distributed in the same pattern as the cholinesterase-positive sites, and were of similar appearance (Fig. 2 a, b; Fig. 3 a to c). In other sections from rabies 1820B virus-infected mice, which were double-stained with the cholinesterase method and the
Fig. 1. Representative motor end plates in cross-sections of leg muscles of mice. The dark areas are the reaction product formed using the Karnovsky-Roche method for detecting acetylcholinesterase, an enzyme which is concentrated at motor end plates.
immunofluorescent technique, some of the cholinesterase-positive sites also showed specific fluorescence. After injection in both footpad and leg muscle, the amount of antigen present was significantly increased. The mean distribution of antigen-positive sites in legs from three specimens at 1 h post-inoculation is given in Table 1. Fluorescent sites of the MEP type were seen only rarely at later times.

Specific fluorescence was not observed in neuromuscular spindles, muscle cells, nerves, spinal cord, spinal roots, or dorsal root ganglia in any sections at 1 h or 6 h post-inoculation. Antigen was consistently seen in either ventral root fibres where they pass through the white matter of the lumbar spinal cord or in ventral horn cells before antigen was found in dorsal roots or dorsal root ganglion cells. Antigen was found in ventral horn cells at 20 h post-inoculation in one section of the spinal cord. At subsequent times, antigen increased in amount and occurred in more widely distributed areas of the grey matter. In one experiment at 28 h post-inoculation, antigen was also detected in one dorsal root ganglion from each of two specimens examined. The sections containing antigen-positive ganglion cells did not have antigen in the grey matter. These ganglion cells were on sections of the spinal cord caudal to sections with antigen-containing ventral horn cells in the spinal cord. The fluorescing ganglion cells and the fluorescing ventral horn cells were separated by at least 30 sections with fluorescence in neither the ganglia nor the spinal cord. At 48 h post-inoculation, antigen was present in two ganglia, each with numerous cells fluorescing. At 60 h post-inoculation, antigen was present in numerous ganglia from different anatomical regions of the spinal cord. Antigen was not found on contralateral ganglia until 72 h post-inoculation at which time there was fluorescence in virtually all of the ipsilateral ganglia. Antigen was seen in axons and in single muscle cells or small foci of muscle cells in scattered places in the muscles at 72 and 96
Fig. 3. Localization of rabies virus antigen at 1 h post-inoculation of adult mice by fluorescent antibody staining; see Fig. 2.
Table 1. Immunochemical localization of rabies 1820B virus in leg muscles of adult mice

<table>
<thead>
<tr>
<th>Time of observation and structure (h post-inoculation)*</th>
<th>Slide number†</th>
<th>MEP‡</th>
<th>MC‡</th>
<th>MS‡</th>
<th>MEP</th>
<th>MC</th>
<th>MS</th>
<th>MEP</th>
<th>MC</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h observations</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 1 h counts are means of three animals; 72 h and 96 h counts are means of two animals.
† There were three serial sections per slide. Sections were 120 μm apart. Slides were numbered from the section nearest knee to the section nearest foot.
‡ MEP, Motor end-plate; MC, muscle cell or focus of muscle cells; MS, muscle spindle.

h post-inoculation and in neuromuscular spindles rarely at 72 h post-inoculation and commonly at 96 h post-inoculation. Antigen configurations resembling MEPs were found on rare occasions at 72 h and 96 h post-inoculation. Since many muscle cells were involved at these times and since antigen was often widespread within these muscle cells, it is possible that these determinations represent underestimations of the number of MEPs at which antigen was located. The numbers of fluorescing MEPs, muscle cells and muscle spindles in the infected
Rabies infection of nerves

Rabies infection of nerves

379

Leg are shown in Table 1. Positive muscle spindles were frequently found at 96 h post-inoculation in areas of muscles where there were no antigen-positive extrafusal muscle fibres. In the contralateral leg isolated antigen-positive sites resembling MEPs were found at 96 h post-inoculation and there were numerous positive spindles by 120 h post-inoculation.

Immunofluorescence was not detected after mice had been injected with BPL-inactivated rabies 1820B virus or with rabies 1820B virus given at the same time as rabies virus antibody. In contrast, when rabies 1820B virus was inoculated 24 h after rabies virus antibody, fluorescence was seen at 1 h post-inoculation in a pattern similar to that observed with the same virus without prior injection of antibody. However, there was no fluorescence in tissues from these mice at 96 h post-inoculation nor did any of these mice held for observation die.

Rabies CVS virus: autoradiography

Labelled CVS virus was detected in longitudinal sections of leg muscles at 6 h post-inoculation (Fig. 4 a). The distribution and shapes of these autoradiographic sites were similar to those of the fluorescent sites seen in sections from mice infected with rabies 1820B virus. Labelled antigen was not found in the spinal cord at 6 h post-inoculation; 1 h specimens were not examined.

Sindbis virus: immunofluorescence and autoradiography

Specific fluorescence was seen in leg sections from only a few of the many Sindbis Ar86 virus-infected mice at 1 and 6 h post-inoculation. Precise definition of the sites at which the antigen was located was impossible by this technique although fluorescent fibres were found in connective tissue and other locations which included the edges of muscle and the junctions of muscle and connective tissue. Sindbis Ar86 virus antigen-positive nerves were observed using autoradiography on leg tissues harvested at 6 h post-inoculation. In one such case, antigen was observed in both the nerve and its associated MEP (Fig. 4 b).

Sections from mice infected with Sindbis Ar339 virus were negative by immunofluorescence at nerve structures at both 1 and 6 h post-inoculation and at 6 h post-inoculation by autoradiography, despite high amounts of antigen input. Tissues from Sindbis virus-infected mice were not examined at times later than 6 h post-inoculation.

Nerve section

No virus antigen was detected by immunofluorescence at nerve sites at 1 h or 96 h post-inoculation in the leg muscles of mice injected with either rabies 1820B virus or Sindbis Ar86 virus 7 days after section of the sciatic nerve. No deaths occurred among the mice kept for observation after denervation and infection with either virus.

Electrical stimulation of the sciatic nerve

Electrical stimulation of the sciatic nerve of mice injected with a high dose of rabies 1820B virus did not obviously affect the distribution of virus antigen in the leg muscles at 0.5 h or at 1 h post-inoculation. Total mortality was unaffected, but stimulated mice had a somewhat longer (7-6 days) average survival time than did mock-stimulated mice (6-1 days). When BPL-inactivated rabies 1820B virus was used in stimulation experiments, only 2 or 3 fluoresenting sites were seen in all the leg sections examined at 1 h post-inoculation. Antigen did not appear at neuromuscular spindles at 96 h post-inoculation and there was no mortality.

Mice injected with Sindbis Ar86 virus, stimulated for 1 h, and kept for observation did not die, whereas 66% of the mock-stimulated, infected mice died. Both stimulated and non-stimulated mice infected with Sindbis Ar339 virus survived.

Intraplantar injection of HRP resulted in the appearance of HRP reaction product at synaptic clefts 1 h later. Electrical stimulation appeared to increase the number of MEPs at
which reaction product was observed although the results could not be quantified with the
techniques used.

**DISCUSSION**

The results presented here provide evidence that the axon terminal at the NMJ is a site of
entry for rabies virus (strains 1820B and CVS) into the nervous system. We use the term
neuromuscular junction (NMJ) to refer to the structural unit consisting of the motor axon
terminal, its Schwann cell covering and the underlying modified portion of the muscle fibre
surface, whereas we use the term motor end-plate (MEP) to denote the area of the muscle
fibre receiving the branches of a single axon (Gauthier, 1976). The MEPs were visualized by
using a standard histochemical technique for the detection of acetylcholinesterase which is in
high concentration there. Soon after injection of virus, rabies-specific fluorescence occurred at
sites with size, morphology and distribution similar to those of MEPs; specific fluorescence
was seen at some of the cholinesterase-positive sites in double-stained sections. In addition,
virus-specific antigen was detected in ventral root fibres and in ventral horn cells sooner after
injection of virus than in dorsal roots, dorsal root ganglia or muscle spindles; that is, it was
detected in motor nerves earlier than in sensory nerves. Fluorescing ventral horn and ganglion
cells were not necessarily in the same cross-section at some early stages of infection although
they may have been in the same anatomical segment. This was not an unexpected result since
the spinal nerves from the hind limbs of the mouse enter the cord at such an angle that ganglia
are located caudal to the level where the axons in the dorsal and ventral roots enter the spinal
cord. In addition, rabies virus apparently spreads rapidly from motor neurons in the lumbar
region to spinal ganglia in other regions of the spinal cord perhaps via intersegmental
collaterals. From these ganglia, there was additional spread to the grey matter. The pattern
we have observed is consistent with that of others (Johnson, 1965) at comparable time
periods.

The temporal sequence in this study indicates that virus travelled initially from the axon
terminal at the NMJ to the spinal cord rather than from spinal cord to axon terminal as would
occur if virus were taken up first at sensory endings. The early appearance of rabies virus
antigen at MEPs was not always associated with death of the host. There was antigen at
MEPs when virus was injected 24 h after administration of anti-rabies serum, but all mice
treated similarly and held for observation survived.

Our conclusion that rabies virus enters the nervous system at the motor axon terminal of
NMJs appears to conflict with the conclusion drawn by Murphy and his co-workers that
virus entry takes place in the sensory endings of muscle and tendon spindles after virus
replication in muscle cells (Murphy et al., 1973a, b; Harrison & Murphy, 1978). However,
the two studies contained both major and minor design differences, including host species,
developmental stages of the host, virus strains and sources, sampling schemes, sites of
injection, and methods of tissue preparation. One or more of these differences could explain
the apparent discrepancies in results and the conclusions drawn from them.

Alternatively, it is possible that there is no conflict in results. In Murphy et al. 1973 (a),
individual dorsal root spinal ganglion cells were shown to be antigen-positive by 60 h after
infection with vampire bat rabies or Mokola viruses and 72 h after infection with Lagos bat
virus. These investigators also report (Murphy et al., 1973 b) that antigen was detectable by
immunofluorescence at 60 h in individual ipsilateral lumbar spinal cord neurons of a few of
the hamsters inoculated i.m. with Mokola, Lagos bat, vampire bat rabies or CVS rabies virus.
Their cumulative results show that definitely in the case of Lagos bat virus, and perhaps in the
case of CVS rabies, antigen reaches spinal cord neurons before reaching sensory neurons
thereby leaving open the possibility of uptake at MEPs.

If rabies virus enters the axon terminal at the NMJ, what is the mechanism of uptake? A
Rabies infection of nerves

A number of macromolecules have been found to be taken up by axon terminals and transported in the retrograde direction to the nerve cell body, but the mechanisms of uptake are varied and range from the non-specific, as with HRP, to the highly selective, as with nerve growth factor (for review, see Kristensson, 1978). Labelled nerve growth factor is taken up by sensory endings after injection into the forepaw of rats and carried by retrograde axonal transport to the dorsal root ganglia (Stoeckel et al., 1975). Conversely, labelled tetanus toxin is found in the centrolateral spinal cord grey matter after i.m. injection, implying uptake by motor neuron endings (Schwab & Thoenen, 1976). Our findings suggest that peripheral nerve endings could display a similar selectivity in the uptake of rabies virus. Vesicles containing HRP have been found in motor axons of murine eye muscles soon after intravenous injection and later in nerve cell bodies in cranial nerve nuclei in the brain stem (Kristensson, 1977). Virus may reach the vicinity of NMJs in a manner similar to the diffusion of HRP into muscle (Heuser & Reese, 1973; Kristensson, 1977), but the accumulation of virus antigen at the MEP apparently requires some virus-specific characteristic, possibly a receptor, since Sindbis Ar339 virus did not accumulate there. The cumulative results of several different experiments indicate that the association of rabies virus with the MEP and the apparent uptake into the axon terminal are events which require both viable rabies virus and intact NMJs. Inactivation of the virus by BPL treatment or mixing the virus with antibody prevented the association of virus with MEps. Alteration of the integrity of the NMJ by nerve section 7 days before inoculation of virus also prevented localization of antigen at MEps. Electrical stimulation, of even a few minutes duration, increases the amount of HRP taken up at the frog NMJ (Litchy, 1973; Heuser & Reese, 1973). In our experiments, uptake of rabies and Sindbis viruses seemed to be delayed or decreased rather than increased after stimulation as would be expected if virus uptake were mediated by ‘HRP-like’ non-specific incorporation into vesicles. However, we cannot eliminate the possibility of vesicular incorporation of virus at the axon endings since small amounts of BPL-inactivated rabies virus were found there after stimulation. Four hypotheses which might explain the observed effects of electrical stimulation are as follows. (i) Stimulation decreases the availability of cell membrane receptors for virus attachment. (ii) Stimulation results in the release from the nerve of a substance which interferes with virus uptake. (iii) Stimulation damages the nerve in a manner which affects virus uptake, replication, or transport but does not affect excitation of the muscle cells. (iv) Increased virus uptake at the axon terminal following stimulation is accompanied by virus release back into the synaptic cleft. The differences observed between the two biotypes of Sindbis virus may account, in part, for their different pathogenetic characteristics in the mouse. A neurotropic strain of Sindbis virus (Ar86) was found at the MEP whereas a non-neurotropic Sindbis strain (Ar339) was not. We have previously presented evidence that these two strains of Sindbis virus do not share the same host cell receptor on cells in culture and that there are significantly more host cell receptors on cultured neuronal cells of spinal origin for the Ar86 strain of Sindbis than for the Ar339 strain (Smith & Tignor, 1980).

The simplest interpretation of the results presented here is that the entry of rabies virus into the nervous system occurs at the motor axon terminal with specific attachment at normal NMJs. However, other possibilities remain. Virus could be taken up at either or both motor and sensory endings but which of these is involved may vary with the virus strain, the host species and its stage of development. None of these possibilities can be eliminated yet, since, in our study, rabies virus antigen was detected at sensory nerves in the inoculated limb before it was detected in the contralateral limb suggesting that uptake into sensory nerves could occur but at a slower rate than into motor nerves.

This study was supported by USPHS grants RO1-AI12541, PO1-AI11132, and T32-NS07036. The research discussions with Dr Thomas L. Lentz and the technical assistance of Ms Katherine Moreno.
and Ms Barbara Collett were invaluable. We appreciate the editorial assistance of Dr Robert E. Shope and Joan Crick.

REFERENCES


(Received 26 January 1981)