Journal of NeuroVirology (1997) 3, 38–48
© 1997 Journal of NeuroVirology, Inc.
http://www.jneurovirol.com

Susceptibility to a neurotropic virus and its changing distribution in the developing brain is a function of CNS maturity

Kevin R Oliver, Martina F Scallan, Heather Dyson and John K Fazakerley

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK

Many major physiological changes occur within the rodent central nervous system (CNS) during the first few postnatal weeks. These include axonogenesis, synaptogenesis and myelination. Concomitant with CNS development over this period, there is a decrease in susceptibility to many neurotropic virus infections in that infection of suckling animals results in lethal encephalitis whereas infection of weanling animals is not lethal. The events underlying this dramatic change in susceptibility have been unclear. Here we demonstrate that age-related virulence of the neurotropic alphavirus, Semliki Forest virus is dependent upon ability of the infection to spread in the CNS. This is not determined by maturity of interferon, or specific immune responses or the blood brain barrier, but by maturity of neuronal systems. Detailed study of the course of infection in the cortex, hippocampus and cerebellum during their postnatal development indicates that as these and other neuronal systems mature they become resistant to spread of the virus and the pattern of infection changes from widespread to focal.

Keywords: neurovirulence; alphavirus; neuronal differentiation; CNS development; neuronal maturation

Introduction

It has been known for a long time that in rodents there is a marked change in susceptibility to neurotropic virus infections during the first few postnatal weeks (Sigel, 1952; Johnson *et al*, 1972; Fleming, 1977; Ogata *et al*, 1991). This age-related susceptibility has been documented with numerous viruses including St Louis encephalitis, West Nile, Sindbis, Japanese encephalitis, Yellow Fever, Semliki Forest, measles and Herpes viruses (Theiler, 1930; O'Leary *et al*, 1942; Lennette and Kowprowski, 1944; Melnick *et al*, 1951; Sigel, 1952; Johnson *et al*, 1972; Fleming, 1977; Swoveland and Johnson, 1989; Ogata *et al*, 1991). In all of these cases, infection of suckling mice results in a lethal encephalitis, whereas infection of weaned animals is not lethal. Although well-documented, the events underlying this dramatic change in susceptibility have for many years remained unclear. Many early investigators considered the phenomenon to be linked to maturity of immune responses. Where this has been investigated it has been found not to be the case (Johnson *et al*, 1972; Griffin, 1976; Fleming, 1977; Fazakerley *et al*, 1993; Schneider-Schaulies *et al*, 1989; Swoveland and Johnson, 1989; Sharpe *et al*, 1990).

One of the most intensely studied examples of age-related neurovirulence is Semliki Forest virus (SFV), an alphavirus of the Togaviridae (Bradish *et al*, 1971; Bradish and Allner, 1972; Pattyn *et al*, 1975; Fleming, 1977; Woodward *et al*, 1978). Neonatal mice inoculated intraperitoneally (ip) with the A7(74) strain of SFV rapidly die from a fulminant encephalitis. In contrast, ip infection of 3–4 week old mice results in a subclinical encephalitis. Following ip inoculation of mice of all ages, a plasma viremia is established from which virus is seeded across cerebral capillaries into the CNS initiating small, perivascular foci of infection (Pusztai *et al*, 1971; Fleming, 1977; Fazakerley *et al*, 1993). In suckling mice these foci rapidly enlarge resulting in a panencephalitis. In 3–4 week old mice the perivascular foci do not enlarge with time, and by 10 days post-infection (PI) no virus can be detected in the brain by infectivity assay or *in situ* hybridization (Fazakerley *et al*, 1993). Clearance of infectious virus from the CNS and the demyelinating encephalomyelitis which develops in 3–4 week old mice are dependent upon specific immune

Correspondence: JK Fazakerley
JK Fazakerley, MF Scallan and H Dyson are presently in the Department of Veterinary Pathology, University of Edinburgh, UK
Received 21 May 1996; revised 8 August 1996; accepted 21 August 1996

responses (Jagelman *et al*, 1978; Fazakerley and Webb, 1987a, b; Subak-Sharpe *et al*, 1993). However, the age-related difference in virus spread and virulence cannot be attributed to maturity of specific immunity since, in 3–4 week old athymic *nu/nu* mice (with no T-lymphocytes) or in 3–4 week old mice with severe combined immunodeficiency (SCID, with no T or B lymphocytes), CNS infection does not become widespread and virus is able to persist in small foci (Fazakerley and Webb, 1987a; Fazakerley *et al*, 1993; Amor *et al*, 1996).

An alternative explanation for SFV age-related neurovirulence is a differential interaction between virus and CNS cells in developing and mature mouse brain. Evidence in support of this comes from electronmicroscopic studies of virus replication which have consistently demonstrated absence of complete replication in infected neurons and oligodendrocytes from 3–4 week old mice, whereas all stages of virus replication are readily detected in infected neurons of neonatal mice (Pathak *et al*, 1976; Pathak and Webb, 1978; Fazakerley *et al*, 1993). In this study we have investigated changes in the mortality, spread, distribution and pathology of SFV A7(74) infection in mouse brain from birth (P0) to 4 weeks of age (P28).

Results

Mortality study

The birth dates of litters were carefully noted and recorded as postnatal day 0 (P0). Litters were inoculated intraperitoneally (ip) with SFV A7(74) at P5, P9, P10, P11, P12, P13, P14, P15 and P25. All mice were given 500 plaque forming units (PFU) of virus. One or more litters were inoculated at each age point, each litter contained three to nine mice. Figure 1 shows the percentage mortality. There was a marked transition in mortality between P12 and P14, from 100% mortality to 100% survival. Between P2 and P12, increasing age at infection correlated with an increase in average day of death.

Age-related changes in the spread and distribution of virus in the brain

P2, P4, P6, P8, P10, P12, P14, P16, P18, P20, and 3–4 week old mice were inoculated ip with 5000 PFU SFV A7(74). Animals were sacrificed at various times post-infection (PI) and the brains removed and processed for *in situ* hybridization to determine the distribution of virus infected cells. Determination of viral RNA-positive cells was considered preferential to detection of virus protein-positive cells since it is clear that replication of RNA viruses in CNS cells may be restricted leading to virus RNA-positive, virus protein-negative cells (Cash *et al*, 1985). At least three brains at each sampling time point for each age at inoculation were studied. The results were highly reproducible. Representative autoradiographic images from each sampling point

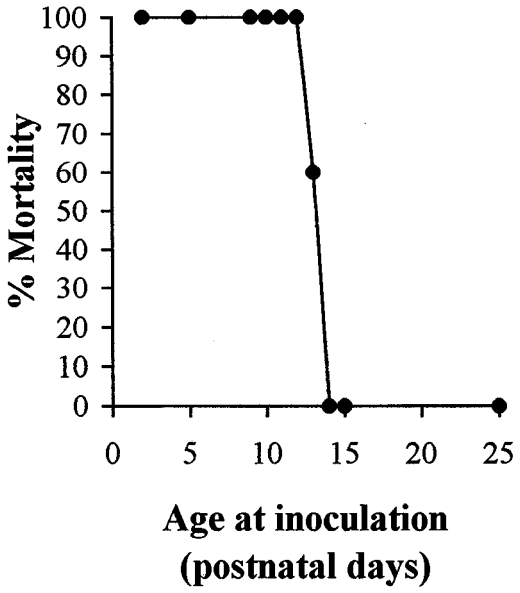

Figure 1 Age-related mortality resulting from SFV A7(74) infection. Note pronounced change from 100% survival to 100% mortality between postnatal days 12 and 14.

for mice infected between P2 and 3–4 weeks of age are shown in Figure 2. SFV RNA was detectable in the brain as early as 16 h in mice inoculated at P2, P4, P6 and P8. Initial foci of infection were often observed in submeningeal regions, commonly in the olfactory bulb and cerebellum. Interestingly, these are amongst the most immature regions of the CNS in the early postnatal period. The time taken for virus to reach the brain increased with age, thus virus could not be detected in the brains of mice inoculated at P20 until 48 h.

An overview of Figure 2 shows that for mice aged P12 or less at inoculation, the original foci rapidly enlarged to produce a widespread infection which was apparent by 36 h to 48 h. In mice aged P14 or greater at time of infection, many small foci of positive cells were apparent in the brain, but generally these did not disseminate into widespread, confluent areas of infection as seen in the younger mice. In addition to this major change in the overall pattern of infection, age-related changes in spread and distribution were observed in several specific systems, particularly the cerebral cortex, hippocampus and cerebellum.

Cortex

It is widely accepted that during development of the cerebral cortex, neuronal progenitors generated in fetal life migrate outward from the ventricular zone along radial glia to their specifically determined final positions (Rakic, 1972, 1988). Successively generated cells push through cells already present in the fetal neocortex and come to lie in more superficial layers (Angevine and Sidman, 1961;

Berry *et al*, 1964; Rakic, 1972, 1974; Tan and Breen, 1993). This radial inside-out formation results in least mature neurons in layer II and most mature neurons in layer I and VI in a cortex which is largely composed of its ontogenic columns (Rakic, 1988).

Age-related changes in the cortex can be seen in Figures 2 and 3. Figure 3 shows representative images from the cortices of animals inoculated at P4, P8, and P12. Virus was detected with a digoxigenin-labeled RNA probe and sections counterstained with haematoxylin and eosin. In animals infected at P2 and P4, viral RNA-positive cells showed distinct columnar infection patterns. The number of columnar foci of infection increased with time post-infection and were predominantly in layers II and III and tapered towards the deeper

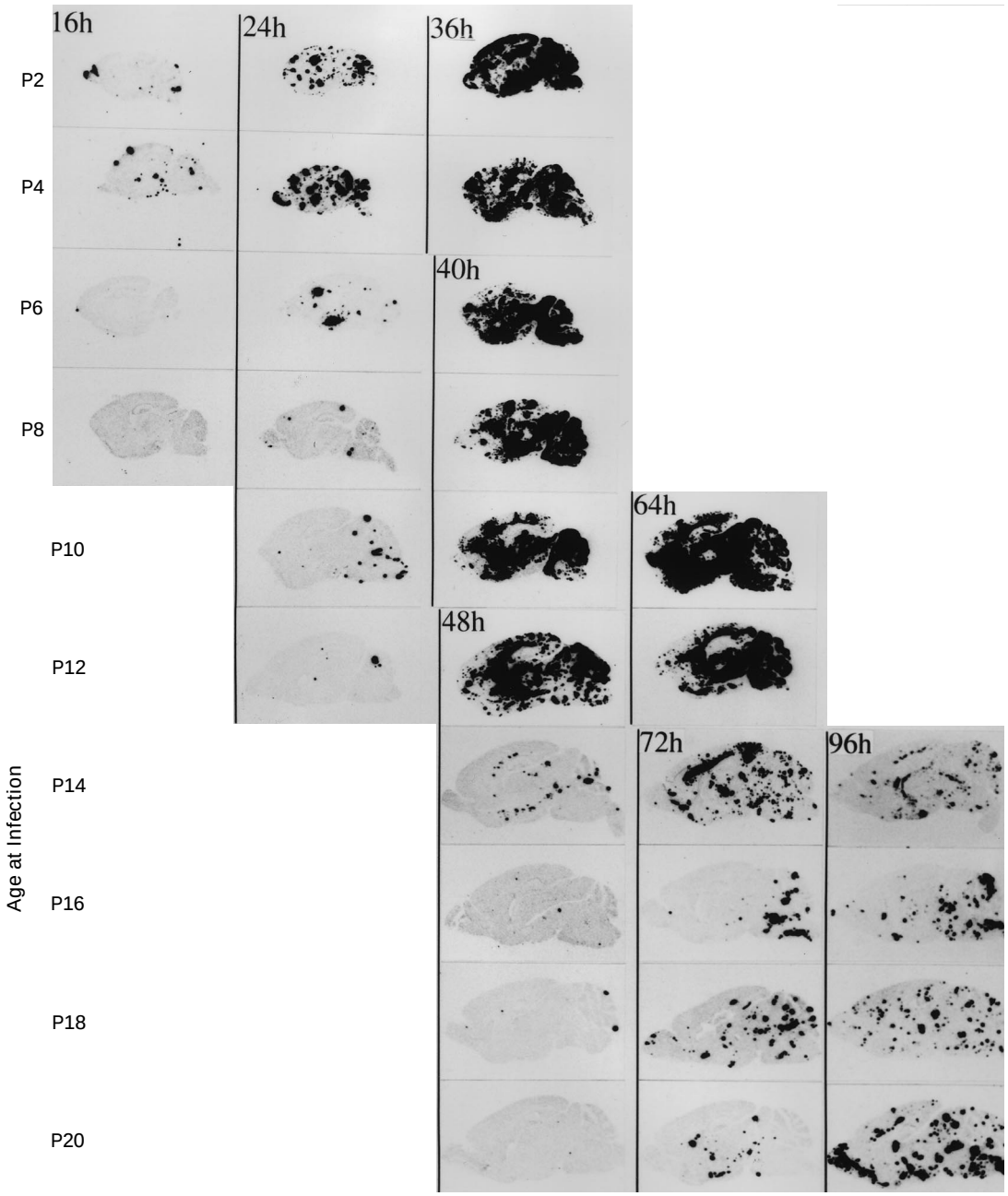

Figure 2 Representative autoradiographic images of sagital sections all cut at the level of the caudate, illustrating the neuroanatomical distribution of SFV RNA-positive cells during mouse brain development. Viral RNA is indicated by black signal. Postnatal age at inoculation (P) is indicated on the left, whilst sampling times post-infection are indicated in the upper left corner. Note the overall change in pattern of infection from widespread to focal between P12 and P14, and the columns of infected cells in the cortices of mice inoculated at P4, sampled at 24 h, and inoculated at P8 or P10 and sampled at 40 h.

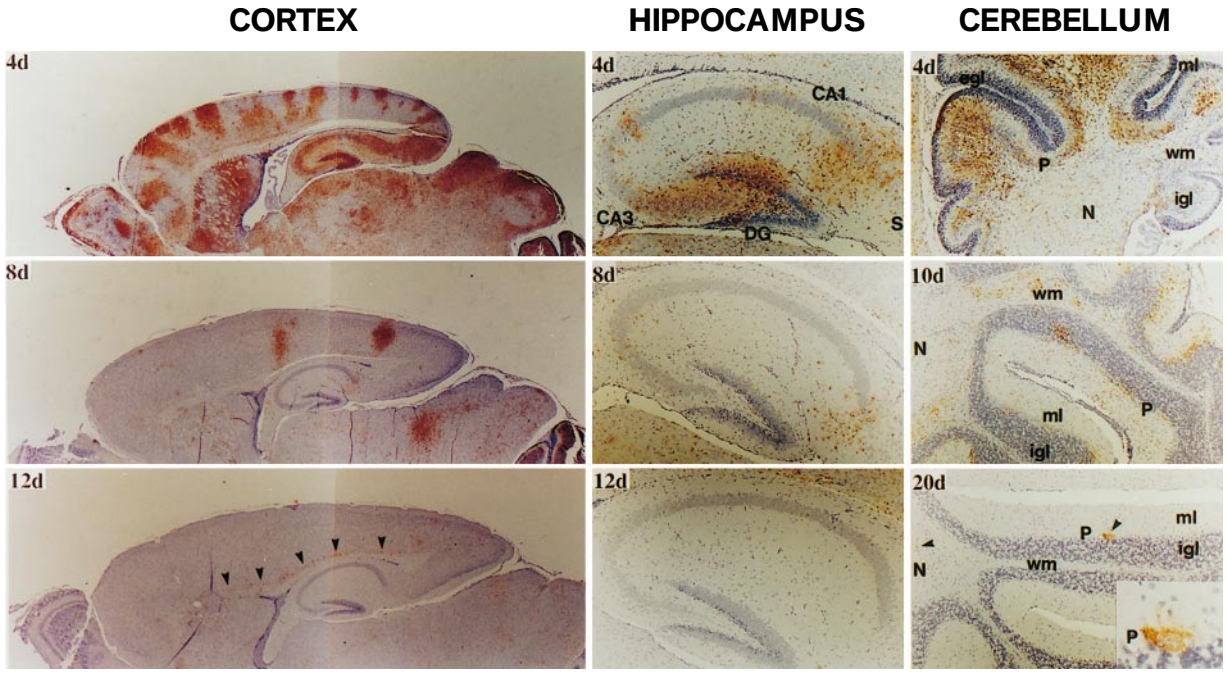

Figure 3 Microscopic analysis of sagittal sections detailing age-related changes in virus distribution in cortex, hippocampus, and cerebellum, as detected by *in situ* hybridization using a digoxigenin-labeled RNA probe. *Cortex*: Bar indicates approximate division of frontal and fore- and hindlimb regions of the cortex and the occipital cortex (left (rostral) of line, and right (caudal) of line, respectively). Note at P4, the numerous columns of virus-positive cells, which are wider in the frontal and narrower in the occipital cortex. These are reduced in number by P8. By P12, only cells in deep layer VI, the cingulate gyrus and the corpus callosum are consistently infected (arrowheads). *Hippocampus*: At P4, note infection of the external limb of the dentate gyrus (DG), CA3, and caudal CA1/subicular neurons consistent with spread of virus along connected pathways. Spread of infection is progressively curtailed with age and is completely absent by P12. *Cerebellum*: There is widespread infection of external and internal granule cell layers (egl, igl), molecular layer (ml), white matter (wm), deep nuclei (N) and Purkinje neurons (P). This is reduced with age (e.g. P10) and confined in older mice (e.g. P20) to occasional scattered Purkinje cells and cells of the connected deep cerebellar nuclei.

layers. Foci in layers V and VI tapered from the corpus callosum towards the outer layers. Columns tended to be broad and overlapping in the prefrontal, frontal, fore- and hindlimb cortical areas and narrow in the occipital cortex (Figure 3). SFV RNA was present in apical dendrites of neurons, passing from layer V and layer III towards layer I (Figures 3 and 4a). There were few infected cells in layer IV.

Animals inoculated at P8 and sampled at 40 h showed much reduced levels of infection and fewer columns of infection. Mice inoculated at P12, and to a lesser extent, P14 showed infection only in the deepest regions of layer VI (Figure 3) and in the underlying cingulate gyrus and corpus callosum. Barrels of infection were apparent only rarely. In the corpus callosum, small cells, possibly oligodendrocytes, were infected. Animals inoculated at P16 and above showed individual or small foci of RNA-positive cells late in infection which were apparently randomly scattered in any cortical layer.

Unlike Japanese encephalitis virus (Ogata *et al*, 1991), the temporal changes in the pattern of SFV RNA distribution did not directly correlate with neuronal ontogeny. The columnar distribution of infection in the cortex, prominent in animals inoculated between P2 and P8, correlates well with the known columnar interconnectivity between neurons in layers II, III and V and the functional organisation of the cortex (Purves *et al*, 1992; Malach, 1994) and indicates that virus spreads along neuronal processes (Figure 4a). The characteristic broad banding of the frontal, fore-and hindlimb cortical areas, and the narrow banding of the occipital cortex (Figure 3), reflect the respective broad and narrow bandings characteristic of motor and sensory neuronal groupings (Purves *et al*, 1992). Infection patterns in P2 and P4 mouse brains are consistent with spread along developing connections during the widespread axonogenesis and synaptogenesis occuring at this time, particularly formation of thalamocortical afferents, other connections to and from subcortical regions and cortical axon collaterals (Eayrs and Goodhead, 1959; Altman, 1971, 1972a; Rees *et al*, 1976; Kristt, 1978; Landis, 1983; Agmon *et al*, 1993).

With increasing age (P4 to P10) fewer columns of infected cells were observed (Figure 3). During this period, cortical connectivity is largely completed and drammatically sculpted by axonal loss (Ivy and Killackey, 1982; Stanfield *et al*, 1982; Bates and Killackey, 1984; Thong and Dreher, 1986). For example, projections to the superior colliculus and spinal cord from layer V neurons in the neonate are

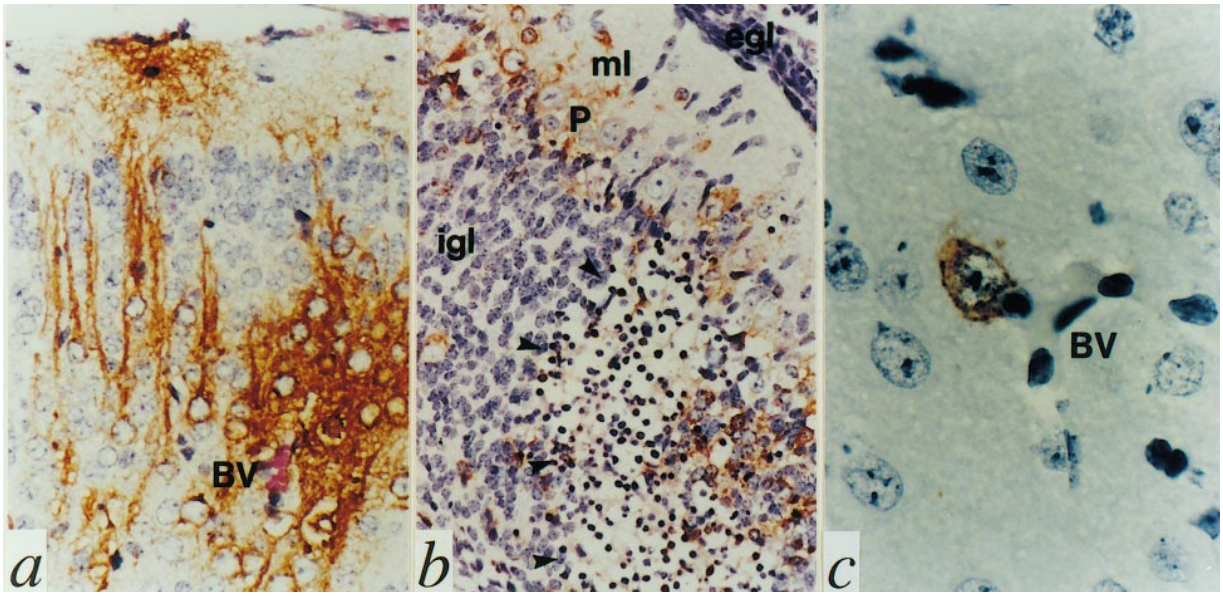

Figure 4 SFV RNA-positive cells with neuronal morphology within a columnar focus of infection in the occipital cortex, 24 h after inoculation of a P4 mouse. Field of view encompasses cortical layers I-III. Note that neuronal processes are positive, particularly the apical dendrites (arrowheads). The blood vessel (BV) present may have been the site of entry of virus into this area with subsequent spread within the barrel along local neuronal connections. (b) Pyknotic nuclei (arrowheads), in the cerebellar internal granule cell layer (igl) of a mouse inoculated at P8 and sampled at 48 h. Purkinje cells (P) and cells in the molecular layer (ml) were also infected. egl, external granule cell layer. (c) Single SFV RNA-positive cell with neuronal morphology adjacent to a blood vessel (BV) in the cortex of a SCID mouse infected at 3–4 weeks of age, sampled at 28 days. Note that the infection has not spread from this to adjacent cells. Note also that virus cannot be detected in the processes of this mature neuron. This pattern of infection is also representative of normal 3–4 week old BALB/c mice and was observed in athymic *nu/nu* mice months(>6) after infection.

distributed along the entire rostrocaudal axis of the cerebral cortex but with increasing age these connections are progressively reduced through axonal loss, so that in the adult they are largely limited to the visual or sensorimotor cortex, respectively. In several of the sections examined this network demonstrated spread of virus along subcortical neuronal pathways. Layer V neurons project to, and receive projections via the internal capsule from, the midbrain, hindbrain and spinal cord (O'Leary and Koester, 1993). This system also demonstrated spread of virus with cells within the internal capsule and connected nuclei, such as the pontine/olivary nuclei (nearby) and the superior colliculus (distant) all positive for viral RNA.

Hippocampus

With increasing age at infection, distinct and reproducible changes in distribution of virus infected cells were observed in the hippocampus (Figure 3). Mice inoculated at P2 and P4 and sampled at 36 h showed almost identical patterns of infection, with granule cells of the external limb of the dentate gyrus, pyramidal neurons of CA3, CA2 and the most caudal part of CA1 and neurons of the subiculum infected (see, for example, P4 mouse in Figure 3). Granule cell processes reaching into the molecular layer, and also processes in the hilus, presumed to be collaterals and mossy fibre projections to the CA3 region, were positive for SFV RNA. Likewise, CA3 pyramidal neuron tufts reaching into the radiatum and lacunosum moleculare layers, as well as the stratum oriens were positive. In contrast, widespread infection of the CA1 region was uncommon, despite infection of the adjacent subicular complex.

Mice inoculated at P6 and sampled at 40 h showed a similar distribution of infection to that seen in mice inoculated at P2 or P4 and sampled at 36 h. In mice inoculated at P8 and sampled at 40 h, intensely SFV RNA-positive cells were much less numerous and usually confined to one defined area with little or no spread. Figure 3 shows strongly SFV RNA-positive cells in the CA1/subiculum, with a small number of weakly positive cells in the connected CA3 region. With the exception of rare, single neurons, cells positive for viral RNA were never observed in the hippocampus in animals inoculated at P12 and older.

The change in infectivity of this system correlates with the time of its maturation. The hippocampal cortex is unique in that most of the afferent inputs and synapses have been described in the rodent (Bayer, 1985; Brown and Zador, 1990). Direct and indirect pathways connect the hippocampal formation and the subiculum with the septal nuclei,

hypothalamus, thalamus, and widespread regions of the cerebral cortex and midbrain reticular formation. In mice inoculated at P2, P4 and to a lesser extent P6, viral RNA-positive cells could be seen in each of these regions and infection extended into the processes of these cells. As the animals aged and the system matured, spread of virus along hippocampal pathways was progressively reduced such that by P8 infected cells were often seen only in initial afferent entry points. This corresponds to the time by which hippocampal connectivity is mostly complete.

Cerebellum
Mice inoculated at P2 to P6 showed infection of cells in all layers of the cerebellum (P4 shown in Figure 3), including Purkinje cells, external and internal granule cells, cells within the forming white matter tracts and molecular layer, and pyramidal neurons of the deep cerebellar nuclei. In mice sampled 48 h after inoculation at P10, most granule cells had migrated from the external to the internal granule cell layer. Completion of the maturation of this system was associated with a dramatic reduction in the number of infected granule cells. The predominant cell types infected at this time were Purkinje neurons, and cells in the white matter tracts and deep cerebellar nuclei. Mice inoculated at P12 showed further limitation of infection with occasional RNA-positive Purkinje cells and cells of the deep cerebellar nuclei and rarely small foci of granule cells. In mice infected at P20 and older the only cells positive for viral RNA were isolated Purkinje neurons and pyramidal cells in the deep cerebellar nuclei (Figure 3).

The cerebellum is a region of the brain which is one of the last to differentiate. As with granule cells of the dentate gyrus and olfactory bulb, cerebellar granule cells are still actively being produced at birth (Altman, 1972a, c). In the first two postnatal weeks these cells migrate from the external to the internal granule cell layer. During this period of migration in which the cells are also undergoing differentiation and synaptogenesis, these cells remained infectable. After completion of migration and establishment of connections by P10, granule cells were rarely infected. Again indicating that change in the ability of these neurons to support virus replication is associated with their maturation.

Purkinje cells receive input from mossy fibres (via granule cells) and climbing fibres (directly). In the mature cerebellum, Purkinje cells receive input from only one climbing fibre originating in the contralateral inferior olive. In the neonate there are inputs from several climbing fibres which are progressively lost in the first few weeks of postnatal life. Coincident with this is a reduction in Purkinje cell apical cone and dendrite growth at P12 and reduction in synaptogenic rate between mossy fibres and granule cells (Larramendi, 1969; Altman, 1972b; Crepel *et al*, 1976; Mariani and Changeux, 1981). In parallel with the maturation of this system the extent of virus infection of Purkinje cells is progressively reduced from infection of the majority of cells in neonates to infection of only a few in 3–4 week old mice. In the latter, infection of small groups of neurons in the deep cerebellar nuclei was a consistent finding. Purkinje cells project to these deep nuclei suggesting that this pathway continues to transmit virus even in older mice.

Infection in 3–4 week old animals
In 3–4 week old animals, cells including neurons remained infectable as evidenced by individual scattered infected cells (Figure 2d). These cells were generally perivascular and infected as a result of virus in the plasma crossing cerebral endothelial cells (Pathak and Webb, 1974; Fazakerley *et al*, 1993). In contrast to spread of infection in neonatal and suckling mice, virus was unable to spread out from these initial foci of CNS infection in these older mice. To rule out the possibility that this in any way reflected changes in the access of virus to the CNS, a group of 3–4 week old mice were inoculated intracerebrally with 5000 PFU of virus. The infection did not spread but remained focal in neurons and the animals survived the infection.

Discussion

The present studies show that following infection with the avirulent A7(74) strain of SFV, mouse age at time of infection determines whether it will succumb to a panencephalitis and die, or survive. The transition between death and survival is sharp. Mice inoculated intraperitoneally (ip) with SFV A7(74) at P12 or below die. Mice inoculated at P14 and above survive and with increasing age show reduced dissemination of virus in the brain until, in 3–4 week old mice infection is confined to the first CNS cells that the virus reaches resulting in small, scattered, predominantly perivascular foci of infection. This age-related susceptibility is not due to maturity of the immune system, as infection of 3–4 week old mice given 8.0 Gy total body irradiation, 3–4 week old athymic *nu/nu* mice or SCID mice, all with impaired specific immune responses, does not result in widespread infection (Fazakerley and Webb, 1987a, b; Fazakerley *et al*, 1993; Amor *et al*, 1996). It is also independent of α/β interferon since the pattern of CNS infection remains unaltered upon infection of 3–4 week old mice with no functional type-I interferon system (Fazakerley *et al*, manuscript in preparation).

Spread of SFV to the CNS along the olfactory nerve has previously been demonstrated (Kaluza *et al*, 1987). The present study demonstrates that this

virus can spread along neuronal connections within the CNS. Clear and consistent changes in the neuroanatomical distribution of the virus were observed with increasing age at infection and the results outlined in some detail the changes occuring in three systems in which neuronal maturation patterns have been extensively studied, the cortex, hippocampus and cerebellum. Whereas, in the youngest mice, each of these systems showed evidence of virus transmission along neuronal connections, transmission was progressively reduced as these pathways matured with age. This is the most detailed characterisation to date of age-related changes in CNS distribution of a neurotropic virus and demonstrates a clear correlation between maturity of neuronal systems and the ability of a virus to spread. Previous studies on lymphocytic choriomeningitis virus have demonstrated that infection of neonatal rats can result in cerebellar hypoplasia depending upon age at infection and that in this system there is an association between neuronal proliferation and virus replication (Monjan *et al*, 1973). In Japanese Encephalitis virus, infection of mouse cerebral cortex neurons correlates with neuronal ontogeny (Ogata *et al*, 1991).

Many changes in both neurons and glial cells occur in the developing rodent brain between P0 and P14. Axonogenesis, synaptogenesis and myelination are mostly completed during this time (Larramendi, 1969; Altman, 1971, 1972b; Rees *et al*, 1976; Landis, 1983). Although a change in susceptibility of glial cells to productive infection could reduce local amplification of the infection and result in a slower spread, the observed changes in SFV distribution must involve changes in neurons, since even in the absence of glial cell amplification, continued productive replication in neurons would still result in widespread infection, particularly in immunodeficient mice. There must therefore, be changes in virus-neuron interactions. These changes could be at the level of virus entry, replication, maturation or release.

One possible explanation is a developmentally regulated change in expression of the virus receptor molecule on neurons. The receptor for SFV remains unknown. The virus has been reported to bind to major histocompatibility molecules (Helenius *et al*, 1978), though this finding remains controversial. The neuronal receptor for the closely related Sindbis virus has been suggested to be developmentally regulated (Ubol and Griffin, 1991). Alternatively, virus may be able to enter immature developing neurons by a non-specific mechanism. Extensive endocytosis occurs at developing post-synaptic membranes (Altman, 1971; Pathak *et al*, 1976; Rees *et al*, 1976; Vaughn and Sims, 1978; Helenius *et al*, 1980). This process ceases upon neuronal maturation and entry thereafter could depend upon specific receptor mediated endocytosis. In this regard it is worth recalling that the A7(74) strain of SFV was derived by serial passage through suckling mouse brain. In the absence of appropriate selective pressures the virus could have lost its ability to bind or, to bind efficiently to its receptor on neurones. Indeed, it is interesting to speculate that non-specific uptake of viruses by developing neurons could explain the ability of the suckling mouse brain to replicate a wide-range of viruses.

Electronmicroscopic studies indicate that SFV A7(74) can infect neurons in the adult mouse brain but that this infection is rarely productive (Pathak and Webb, 1978, 1988; Fazakerley *et al*, 1993). A second stage at which a developmentally related change could act would be in the ability of neurons to replicate virus. As with many viruses, replication of SFV RNA, maturation and budding are intimately associated with, and demonstrate an absolute requirement for continual smooth membrane production and phospholipid synthesis as has been demonstrated both by electronmicroscopical and biochemical studies (Acheson and Tamm, 1967; Grimley *et al*, 1968, 1970, 1972; Friedman *et al*, 1972; Pathak *et al*, 1976; Froshauer *et al*, 1988; Pathak and Webb, 1978, 1988; Peranen and Kaarianen, 1991; Perez *et al*, 1991; Fazakerley *et al*, 1993). This requirement is also observed with several other viruses (Dales and Siminovitch, 1961; Amako and Dales, 1967; Caliguri and Tamm, 1970; Schlesinger and Malfer, 1982; Katoh *et al*, 1986; Kuge *et al*, 1989; Guinea and Carrasco, 1990). Membrane synthesis is actively ongoing in immature neurons undergoing axonogenesis and synaptogenesis, processes which primarily occur in the first two postnatal weeks (Altman, 1971, 1972b; Rees *et al*, 1976; Landis, 1983). The virus may be able to utilise for replication and, or maturation the smooth membrane vesicles or associated biochemical pathways involved in axonogenesis/synaptogenesis resulting in complete virus replication. With neuronal maturation this process ceases (Altman, 1972b; Rees *et al*, 1976). The absence of suitable membranes or associated biochemical pathways could restrict virus replication or maturation resulting in the observed switch from widespread to focal infection.

Whether neuronal maturation changes act at the level of viral entry, replication or maturation on membranes or at some other point, the mechanism clearly does not apply to the virulent L10 strain. The L10 strain of SFV replicates completely and productively and spreads rapidly in the brains of both neonatal and adult mice (Pathak and Webb, 1976, 1978, Fazakerley *et al*, 1993). There are many base changes between these two viruses both in the nonstructural and more frequently the structural genes (Garoff *et al*, 1980; Takkinen, 1986; Glasgow *et al*, 1994; Santagati *et al*, 1994, 1995). Studies of recombinant viruses should be able to genetically map this difference.

Materials and methods

Mice
BALB/c mice of various defined ages and pregnant females were obtained from OLAC (UK) or the Department of Pathology Animal Unit (University of Cambridge, UK). Pregnant mice were checked twice daily to determine the time of birth of litters.

Virus
The A7(74) strain of Semliki Forest virus (SFV) was as described previously (Fazakerley *et al*, 1993). For use, virus was diluted in phosphate buffered saline with 0.75% bovine serum albumin (PBSA). Stocks were stored at −80°C.

In situ *hybridization*
To determine virus distribution in the CNS, mice were sacrificed and brains rapidly removed, divided sagittally then immersion-fixed in 10% phosphate-buffered formal saline. Fixed brains were embedded in paraffin wax and 5 μm sections cut onto BioBond (British BioCell International) coated slides and processed for *in situ* hybridization to determine distribution of viral RNA.

The *in situ* hybridization method was based on that previously published (Fazakerley *et al*, 1991, 1993). Briefly, sections were dewaxed in xylene, dehydrated and treated sequentially with 0.2 M HCl (20 min), 1% Triton X-114 (1.5 min), 10 μg/ml proteinase K in 20 mM Tris, 2 mM $CaCl_2$, pH 7.0 (30 min, 37°C) and 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 (10 min). Sections were then dehydrated and prehybridized for 1 h at 55°C in hybridization solution containing no dextran sulphate. The hybridization solution consisted of 50% deionised formamide, 5 × Denhardt's solution, 10% dextran sulphate, 0.1% SDS, 250 μg/ml sonicated salmon sperm DNA, 200 μg/ml yeast tRNA, 10 U/ml heparin, pH 6.8. Slides were drained of prehybridization solution and 25 μl hybridization solution containing 2×10^5 cpm/μl of ^{35}S-labeled riboprobe and 10 mM DTT was added to each slide. For *in situ* hybridization with digoxigenin-labeled probes, 25 μl of hybridization solution were added to each slide at a 1/100 dilution of purified transcription product.

The probe was an ^{35}S- or digoxigenin-labeled RNA produced by *in vitro* transcription from pGEM1-SFV (a kind gift of Dr H Garoff, Karolinska Institute, Sweden). This construct, which contained the viral structural genes was linearised at the *Hinc*II site, 5′ of the capsid gene, and transcribed from the T7 promoter, 3′ of the E1 gene. ^{35}S- or digoxigenin-labeled transcripts were produced using a riboprobe Gemini kit (Promega), or a digoxigenin RNA labelling kit (Boehringer-Mannheim). The resulting negative sense transcripts of approximately 4 kb were alkaline hydrolysed to smaller fragments in 0.04 M $NaHCO_3$ buffer (30 min, 60°C). Probes were ethanol-precipitated and resuspended in water.

Hybridization was carried out overnight at 55°C. Sections were washed in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6 (20 min, 37°C), and then in fresh buffer containing 20 μg/ml RNAase A (30 min, 37°C). Sections were rewashed in the above buffer (4 × 15 min, 37°C), then in 2 × SSC, (SSC is 0.15 M NaCl, 0.015 M Na citrate) (2 × 15 min, 37°C) and finally in 0.2 × SSC (2 × 15 min, 55°C). Sections were dehydrated and air dried, and for those hybridized with ^{35}S-labeled probes, autoradiographic images were produced by exposure to Hyperfilm βmax (Amersham, UK).

After the final dehydration, sections hybridized with digoxigenin-labeled probes were placed in 0.3% H_2O_2 in methanol (30 min) to quench any endogenous peroxidase activity, rehydrated, and washed in PBS. Slides were then blocked in 2% Normal Rabbit Serum (NRS) in 0.3% Triton X-114 in 0.1 M Tris, 0.15 M NaCl, pH 7.5 (30 min). Hybridized probe was detected using a sheep anti-digoxigenin antibody (Boehringer Mannheim), followed by a biotinylated rabbit anti-sheep antibody amplified with avidin-biotin-peroxidase complexes (Vector Laboratories) and visualised using diaminobenzidine. Slides were counterstained with haematoxylin and eosin and mounted.

Control sections from infected brains were hybridized with ^{35}S- or digoxigenin-labeled riboprobes specific for the 1D gene of the BeAn strain of Theiler's murine encephalomyelitis virus, another neurotropic positive sense RNA virus used in the laboratory. These probes did not give any signal on sections of SFV infected brains, and SFV-specific probes did not give any signal on sections of Theiler's virus infected tissues.

Acknowledgements

We are very grateful to Dr B Everitt (Department of Anatomy, Cambridge, UK) for help with the neuroanatomical aspects of this study and to Barry Potter for technical assistance. This study was supported by grants from the Medical Research Council and the Myalgic Encephalomyelitis Association.

References

Acheson NH, Tamm I (1967). Replication of Semliki Forest virus: an electron microscopic study. *Virology* **32:** 128–143.

Agmon A, Yang LT, O'Dowd DK, Jones EG (1993). Organized growth of thalamocortical axons from the deep tier of terminations into layer IV of developing mouse barrel cortex. *J Neurosci* **13:** 5365–5382.

Altman J (1971). Coated vesicles and synaptogenesis. A developmental study in the cerebellar cortex of the rat. *Brain Res* **30:** 311–322.

Altman J (1972a). Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J Comp Neurol* **145:** 353–398.

Altman J (1972b). Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. *J Comp Neurol* **145:** 399–464.

Altman J (1972c). Postnatal development of the cerebellar cortex in the rat. III. Maturation of the components of the granular layer. *J Comp Neurol* **145:** 465–514.

Amako K, Dales S (1967). Cytopathology of Mengovirus infection. II. Proliferation of membranous cisternae. *Virology* **32:** 201–215.

Amor S, Scallan MF, Morris MM, Dyson H, Fazakerley JK (1996). Role of the immune response in protection and pathogenesis during Semliki Forest virus encephalitis. *J Gen Virol* **77:** 281–291.

Angevine JB, Sidman RL (1961). Autoradiographical study of cell migration during histogenesis in cerebral cortex of the mouse. *Nature (London)* **192:** 766–768.

Bates CA, Killackey HP (1984). The emergence of a discretely distributed pattern of corticospinal projection neurons. *Dev Brain Res* **13:** 265–273.

Bayer SA (1985). Hippocampal region, In: *The Rat Nervous System, Vol. 1*. Paxinos G, (ed.). Academic Press Inc: Australia, pp 335–348.

Berry M, Rogers AW, Eayrs JT (1964). Pattern of cell migration during cortical histogenesis. *Nature* **203:** 591–593.

Bradish CJ, Allner K (1972). The early responses of mice to respiratory or intraperitoneal infection by defined virulent and avirulent strains of Semliki Forest virus. *J Gen Virol* **15:** 205–218.

Bradish CJ, Allner K, Maber MB (1971). The virulence of original and derived strains of Semliki Forest virus for mice, guinea-pigs and rabbits. *J Gen Virol* **12:** 141–162.

Brown TH, Zador AM (1990). Hippocampus. In: *The Synaptic organisation of the brain*. Shepherd GM, (ed.). Oxford University Press: Oxford, pp 346–388.

Caliguri LA, Tamm I (1970). The role of cytoplasmic membranes in poliovirus biosynthesis. *Virology* **42:** 100–111.

Cash E, Chamorro M, Brahic M (1985). Theiler's virus RNA and protein synthesis in the central nervous system of demyelinating mice. *Virology* **144:** 290–294.

Crepel F, Mariani J, Delhaye-Bouchard N (1976). Evidence for a multiple innervation of Purkinje cells by climbing fibres in the rat cerebellum. *J Neurobiol* **7:** 567–578.

Dales S, Siminovitch L (1961). The development of vaccinia virus in Earl's strain cells as examined by electron microscopy. *J Biophys Biochem Cytology* **10:** 475–500.

Eayrs JT, Goodhead B (1959). Postnatal development of the cerebral cortex in the rat. *J Anat* **93:** 385–402.

Fazakerley JK, Southern P, Bloom F, Buchmeier M (1991). High resolution in situ hybridization to determine the cellular distribution of lymphocytic choriomeningitis virus RNA in the tissues of persistently infected mice: Relevance to arenavirus disease and mechanisms of viral persistence. *J Gen Virol* **72:** 1611–1625.

Fazakerley JK, Pathak S, Scallan M, Amor S, Dyson H (1993). Replication of the A7(74) strain of Semliki Forest virus is restricted in neurons. *Virology* **195:** 627–637.

Fazakerley JK, Webb HE (1987a). Semliki Forest virus-induced, immune-mediated demyelination: Adoptive transfer studies and viral persistance in Nude mice. *J Gen Virol* **68:** 377–385.

Fazakerley JK, Webb HE (1987b). Semliki Forest virus induced, immune mediated demyelination: the effect of irradiation. *Br J Exp Path* **68:** 101–113.

Fleming P (1977). Age-dependent and strain-related differences of virulence of Semliki Forest virus in mice. *J Gen Virol* **37:** 93–105.

Friedman RM, Levin JG, Grimley PM, Berezesky IK (1972). Membrane-associated replication complex in arbovirus infection. *J Virol* **10:** 504–515.

Froshauer S, Kartenbeck J, Helenius A (1988). Alphavirus RNA replication occurs on the cytoplasmic surface of endosomes and lysosomes. *J Cell Biol* **107:** 2075–2086.

Garoff H, Frischauf A-M, Simons K, Lehrach H, Delius H (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature* **288:** 236–241.

Glasgow GM, Killen HM, Liljestrom P, Sheahan BJ, Atkins GJ (1994). A single amino acid in the E2 spike protein of a virulent strain of Semliki Forest virus attenuates pathogenicity. *J Gen Virol* **75:** 663–668.

Griffin DE (1976). Role of the immune response in age-dependent resistance of mice to encephalitis due to Sindbis virus. *J Infect Dis* **133:** 456–464.

Grimley PM, Berezesky IK, Friedman RM (1968). Cytoplasmic structures associated with an Arbovirus infection: Loci of viral ribonucleic acid synthesis. *J Virol* **2:** 1326–1338.

Grimley PM, Friedman RM (1970). Development of Semliki Forest virus in mouse brain - An electron microscopic study. *Exp Molec Path* **12:** 1–13.

Grimley PM, Levin JG, Berezesky IK, Friedman RM (1972). Specific membranous structures associated with the replication of group A Arboviruses. *J Virol* **10:** 492–503.

Guinea R, Carrasco L (1990). Phospholipid biosynthesis and poliovirus genome replication, two coupled phenomena. *EMBO J* **9:** 2001–2016.

Helenius A, Morein B, Simons K, Robinson P, Schirrmacher V, Trhorst C, Strominger JL (1978). Human (HLA-A and HLA-B) and murine (H2-K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. *Proc Natl Acad Sci USA* **75:** 3846–3850.

Helenius A, Marsh M, White J (1980). The entry of viruses into animal cells. *Trends Biochem Sci* **5:** 104–106.

Ivy GO, Killackey HP (1982). Ontogenetic changes in the projections of neocortical neurons. *J Neurosci* **2:** 735–743.

Jagelman S, Suckling AJ, Webb HE, Bowen ETW (1978). The pathogenesis of avirulent Semliki Forest virus infections in athymic nude mice. *J Gen Virol* **41:** 599–607.

Johnson RT, McFarland HF, Levy SE (1972). Age-dependent resistance to viral encephalitis: Studies of infections due to Sindbis virus in mice. *J Infect Dis* **125:** 257–262.

Kaluza G, Lell G, Reinacher M, Stitz L, Willems WR (1987). Neurogenic spread of Semliki Forest virus in mice. *Arch Virol* **93:** 97–110.

Katoh I, Yoshinaka Y, Luftig RB (1986). The effect of cerulenin on Moloney murine leukemia virus morphogenesis. *Virus Res* **5:** 265–276.

Kristt DA (1978). Neuronal differentiation in somatosensory cortex of the rat. I. Relationship to synaptogenesis in the first postnatal week. *Brain Res* **150:** 467–486.

Kuge O, Akamatsu Y, Nishijima M (1989). Abortive infection with Sindbis virus of a Chinese hamster ovary cell mutant defective in phosphatidylserine and phosphatidylethanolamine biosynthesis. *Biochim Biophys Acta* **986:** 61–69.

Landis SC (1983). Neuronal growth cones. *Ann Rev Physiol* **45:** 567–580.

Larramendi LMH (1969). Analysis of synaptogenesis in the cerebellum of the mouse, In: *Neurobiology of Cerebellar Evolution and Development*. Linas R, (ed.). AMA Educ. and Res.Fdn: Chicago, pp 803–843.

Lennette EH, Koprowski H (1944). Influence of age on the susceptibility of mice to infection with certain neurotropic viruses. *J Immunol* **49:** 175–191.

Malach R (1994). Cortical columns as devices for maximizing neuronal diversity. *Trends Neurosci* **17:** 101–105.

Mariani J, Changeux J-P (1981). Ontogenesis of olivocerebellar relationships. I. Studies by intracellular recordings of the multiple innervation of Purkinje cells by Climbing fibres in the developing rat cerebellum. *J Neurosci* **1:** 696–702.

Melnick JL, Paul JR, Riordan JT, Barnett VH, Goldblum N, Zabin E (1951). Isolation from human sera in Egypt of a virus apparently identical to West Nile virus. *Proc Soc Exptl Biol Med* **77:** 661–665.

Monjan AA, Cole GA, Gilden DH, Nathanson N (1973). Pathogenesis of cerebellar hypoplasia produced by lymphocytic choriomeningitis virus infection of neonatal rats. I. Evolution of disease following infection at 4 days of age. *J Neuropath Exp Neurol* **32:** 110–124.

Ogata A, Nagashima K, Hall WW, Ichikawa M, Kimura-Kuroda J, Yasui K (1991). Japanese Encephalitis virus neurotropism is dependent on the degree of neuronal maturity. *J Virol* **65:** 880–886.

O'Leary DDM, Koester SE (1993). Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex. *Neuron* **10:** 991–1006.

O'Leary JL, Smith MG, Reames HR (1942). Influence of age on susceptibility of mice to St Louis encephalitis virus and on the distribution of lesions. *J Exp Med* **75:** 233–247.

Pathak S, Webb HE (1974). Possible mechanisms for the transport of Semliki Forest virus into and within mouse brain: An electron microscopic study. *J Neurol Sci* **23:** 175–184.

Pathak S, Webb HE (1988). Cytoplasmic viral core aggregates and budding of mature virus and spherules in mouse brain following Semliki Forest virus infections. *Inst Phys Conf Ser No. 93* **3** (Chapt. 9): 213–214.

Pathak S, Webb HE, Oaten SW, Bateman S (1976). An electron microscopic study of the development of virulent and avirulent strains of Semliki Forest virus in mouse brain. *J Neurol Sci* **28:** 289–300.

Pathak S, Webb HE (1978) An electron-microscopic study of avirulent and virulent Semliki Forest virus in the brains of different ages of mice. *J Neurol Sci* **39:** 199–211.

Pattyn SR, De Vleesschauwer L, Van Der Groen G (1975). Replication of arboviruses in mouse organ cultures. II. Multiplication of virulent and avirulent Semliki Forest and Western equine encephalitis viruses in mouse organ cultures. *Arch Virol* **49:** 33–37.

Peranen J, Kaarianen L (1991). Biogenesis of Type I Cytopathic vacuoles in Semliki Forest virus-infected BHK cells. *J Virol* **65:** 1623–1627.

Perez L, Guinea R, Carrasco L (1991). Synthesis of Semliki Forest virus RNA requires continuous lipid synthesis. *Virology* **183:** 74–82.

Purves D, Riddle DR, LaMantia A-S (1992). Iterated patterns of brain circuitry (or how the cortex gets its spots). *Trends Neurosci* **15:** 632–638.

Pusztai R, Gould EA, Smith H (1971). Infection patterns in mice of an avirulent and virulent strain of Semliki Forest virus. *Br J Exp Path* **52:** 669–677.

Rakic P (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol* **145:** 61–84.

Rakic P (1974). Neuron in rhesus monkey visual cortex: Systematic relation between time of origin and eventual disposition. *Science* **183:** 425–427.

Rakic P (1988). Specification of cerebral cortical areas. *Science* **241:** 170–176.

Rees RP, Bunge MB, Bunge RP (1976). Morphological changes in the neuritic growth cone and target neuron during synaptic junction development in culture. *J Cell Biol* **68:** 240–263.

Santanagi MG, Maatta JA, Itaranta PV, Salmi AA, Hinkannen AE (1995). The Semliki Forest virus E2 gene as a virulence determinant. *J Gen Virol* **76:** 47–52.

Santanagi MG, Itaranta PV, Koskimies PR, Maatta JA, Salmi AA, Hinkannen AE (1994). Multiple repeating motifs are found in the 3'-terminal non-translated region of Semliki Forest virus-A7 variant genome. *J Gen Virol* **75:** 1499–1504.

Schlesinger MJ, Malfer C (1982). Cerulenin blocks fatty acid acylation of glycoproteins and inhibits Vesicular Stomatitis and Sindbis virus particle formation. *J Biol Chem* **257:** 9887–9890.

Schneider-Schaulies S, Liebert UG, Baczko K, Cattaneo R, Billeter M, ter Meulen V (1989). Restriction of measles virus gene expression in acute and subacute encephalitis of Lewis rats. *Virology* **171:** 525–534.

Sharpe AH, Hunter JJ, Chassler P, Jaenisch R (1990). Role of abortive retroviral infection of neurons in spongiform CNS degeneration. *Nature* **346:** 181–183.

Sigel MM (1952). Influence of age on susceptibility to virus infections with particular reference to laboratory animals. *Annu Rev Microbiol* **6:** 247–280.

Stanfield BB, O'Leary DDM, Fricks C (1982). Selective collateral elimination in early postnatal development restricts cortical distribution of rat pyramidal tract axons. *Nature* **298:** 371–373.

Subak-Sharpe I, Dyson H, Fazakerley JK (1993). In vivo depletion of $CD8^+$ T cells prevents lesions of demyelination in Semliki Forest virus infection. *J Virol* **67:** 7629–7633.

Swoveland PT, Johnson KP (1989). Host age and cell type influence measles virus protein expression in the central nervous system. *Virology* **170:** 131–138.

Takkinen K (1986). Complete nucleotide sequence of the non-structural protein genes of Semliki Forest virus. *Nuc Acids Res* **14:** 5667–5682.

Tan S-S, Breen S (1993). Radial mosaicism and tangential cell dispersion both contribute to mouse neocortical development. *Nature* **362:** 638–640.

Theiler M (1930). Studies on the action of Yellow Fever virus in mice. *Ann Trop Med Parasitol* **24:** 249–272.

Thong IG, Dreher B (1986). The development of the corticotectal pathway in the albino rat. *Dev Brain Res* **25:** 227–238.

Ubol S, Griffin D (1991). Identification of a putative alphavirus receptor on mouse neural cells. *J Virol* **65:** 6913–6921.

Vaughan JE, Sims TJ (1978). Axonal growth cones and developing axonal collaterals form synaptic junctions in embryonic mouse spinal cord. *J Neurocytol* **7:** 337–363.

Woodward CG, Marshall ID, Smith H (1978). Investigations of reasons for the avirulence of the A7 strain of Semliki Forest virus in adult mice. *Br J Exp Path* **58:** 616–624.