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Semliki Forest virus A7(74) transduces hippocampal neurons and glial cells in a temperature-dependent dual manner

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In central nervous system (CNS) tissue preparations, wild-type Semliki Forest virus (SFV) mainly infects neurons, and *in vivo* it causes lethal encephalitis in neonatal and adult rodents. The SFV strain A7(74), by contrast, is avirulent in adult rodents, triggering only limited CNS infection. To examine A7(74) infection in hippocampal tissue, the authors constructed a replicon, termed SFV(A774nsP)-GFP, expressing green fluorescent protein. The results were compared to replication-proficient recombinant A7(74) encoding GFP, named VA7-EGFP. As nonstructural gene mutations can confer temperature sensitivity, the authors also tested whether infection was temperature-dependent. Indeed, at 31°C both viral recombinants transduced significantly more baby hamster kidney cells than at 37°C. When rat hippocampal slices and dissociated cells were incubated at 37°C, SFV(A774nsP)-GFP transduced glial cells but virtually no neurons—the opposite of conventional SFV. For VA7-EGFP at 37°C, the preferred GFP-positive cells in hippocampal slices were also non-neuronal cells. At 31°C, however, a more wild-type phenotype was found, with 33% and 94% of the GFP-positive cells being neurons for SFV(A774nsP)-GFP in slices and dissociated cells, respectively, and 94% neurons for VA7-EGFP in slices. Immunochemical and electrophysiological analyses confirmed that at 37°C virtually all cells transduced by SFV(A774nsP)-GFP in slices were astrocytes, while at 31°C they also contained neurons. These results show that in addition to the developmental age, the temperature determines which cell type becomes infected by A7(74). Our data suggest that A7(74) is avirulent in adult animals because it does not readily replicate in mature neurons at body temperature, whereas it still does so at lower temperatures. *Journal of NeuroVirology* (2003) **9,** 16–28.

Keywords: alphavirus; astrocyte; interneuron; primary culture; pyramidal cell; slice culture

Introduction

Semliki Forest virus (SFV) is an enveloped, positive-strand RNA virus from the alphavirus genus of the togavirus family that infects cells via the major

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histocompatibility class I molecule and other receptors (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 2001). Vectors of both SFV and the closely related Sindbis virus (SIN) have been developed to express high levels of foreign genes *in vitro* and *in vivo* (Liljeström and Garoff, 1991; Bredenbeek *et al*. 1993). In a common approach, the heterologous gene in the "vector RNA" replaces the viral structural protein genes. Vector RNAs are self-replicating and referred to as "replicons." They must be cotransfected with defective "helper RNA" to be packaged into infectious particles. Defective helper RNAs have a large deletion in the genes for the nonstructural proteins 1 to 4 (*nsP1–4*) and are not self-replicating, but can be replicated and transcribed by the nsPs provided by the replicons. Helper subgenomic RNAs are translated to provide the capsid and spike proteins for particle assembly under conditions in which helper RNAs are not packaged. Packaged replicons derived by this approach will infect target cells, but no infectious particles are formed because the replicon genome lacks the structural protein genes.

Wild-type SFV and SIN target neurons in the central nervous system (CNS), induce neuronal apoptosis, and cause encephalitis in mice, sometimes with lethal outcome (Griffin, 2001). Data from studies in mice indicate that neurotropism and neuronal survival are determined by both viral strain and age of the injected animals. Although all strains cause fatal disease in newborn and suckling rodents, the strains SFV A7, its derivative SFV A7(74), and SIN AR339 variants are avirulent for adult animals and cause limited CNS infection, which is cleared within 7 to 10 days post infection (Bradish *et al*, 1971; Pusztai *et al*, 1971; Fazakerley *et al*, 1993; McKnight *et al*, 1996; Oliver *et al*, 1997; Griffin, 1998). Electron-microscopic studies have shown that A7(74) can infect neurons in the adult brain, but this infection is rarely productive (Pathak and Webb, 1978; Fazakerley *et al*, 1993). Thus, infection of adult rodents by A7 and A7(74) is asymptomatic per se, but it leads to immune-mediated, nonlethal CNS demyelination (Kelly *et al*, 1981; Amor *et al*, 1996). Although single–amino acid changes in the spike proteins can alter alphaviral virulence (Davis *et al*, 1986; Lustig *et al*, 1988; Glasgow *et al*, 1994; McKnight *et al*, 1996; Bernard *et al*, 2000), a more recent report employing chimeric virus based on the cloned genomes of A7(74) and the virulent, wild-type strain SFV4 shows that the nonstructural genes, in particular *nsP3*, rather than structural genes, determine avirulence for A7(74) (Tuittila *et al*, 2000). Previous studies have identified mutations in nsP1 and nsP2 to reduce alphaviral cytotoxicity and virulence (Rikkonen, 1996; Dryga *et al*, 1997; Agapov *et al*, 1998; Lundstrom *et al*, 2003; Perri *et al*, 2000; Heise *et al*, 2000; Fazakerley *et al*, 2002).

Wild-type SFV vectors preferentially transduce neurons in CNS tissue cultures, i.e., organotypic rat hippocampal slices (Ehrengruber *et al*, 1999, 2001), and multiply to higher titers in dissociated rat neurons versus glial cells (Atkins *et al*, 1990). The strains A7 and A7(74), by contrast, infect and replicate in glial cells rather than neurons both *in vitro* under "normal" culturing conditions, i.e., at 37°C, and *in vivo* (Bruce *et al*, 1984; Gates *et al*, 1985; Atkins *et al*, 1990). Compared to wild-type SFV4, A7(74) possesses a number of differences in the nonstructural genes (Tuittila *et al*, 2000), and specific mutations in *nsP1-4* can confer temperature sensitivity to SIN (Hahn *et al*, 1989a, 1989b; Boorsma *et al*, 2000) and SFV (Lundstrom *et al*, 2001). Indeed, previous experiments revealed that A7 RNA synthesis and viral growth is temperature-sensitive in baby hamster kidney 21 (BHK) cells and brain tissue cultures. A 1.3- to 1.8-fold higher viral titer was obtained at 37°C than at 39°C (Woodward and Smith, 1979; Hearne *et al*, 1987), and a 17-fold elevated viral RNA synthesis was observed in BHK cells (Hearne *et al*, 1987). The effect was more pronounced at 30°C, where the viral titer and RNA synthesis were 3.3- and 22-fold higher, respectively, as compared to 39°C (Hearne *et al*, 1987). Although it is known that A7 and A7(74) infect and replicate in glial cells rather than neurons, and that A7 replication is temperature-sensitive, a correlation between temperature sensitivity and preferred cell type for infection and replication had not been demonstrated.

In this study, we examined the temperature-dependent transduction patterns for A7(74) in rat hippocampal slice and cell cultures, aiming to distinguish neurons from other cells. Although previous studies employed immunocytochemistry to detect cell type–specific markers (e.g., galactocerebroside for oligodendrocytes (Atkins *et al*, 1990)), we employ here viral recombinants expressing the green fluorescent protein (GFP) reporter molecule to visualize and identify transduced cells. Based on the morphology, location, and electrophysiological as well as immunoreactive properties of GFP-expressing cells in our *in vitro* preparations, we were able to distinguish neuronal from non-neuronal (glial) cells. We analyzed (i) an SFV replicon carrying the genetic loci responsible for the natural A7(74) avirulence, and (ii) a replication-proficient virus based on the full A7(74) genome (Figure 1).

Results

Temperature-dependent GFP expression in BHK cells

Several mutations in nsP1–4 have been described to confer temperature-sensitivity to alphaviral replication and expression (Hahn *et al*, 1989a, 1989b; Boorsma *et al*, 2000; Lundstrom *et al*, 2001). Because *nsP1–4* of the avirulent SFV strain A7(74) contain a number of changes compared to the virulent SFV4 (Tuittila *et al*, 2000), upon which the SFV vector

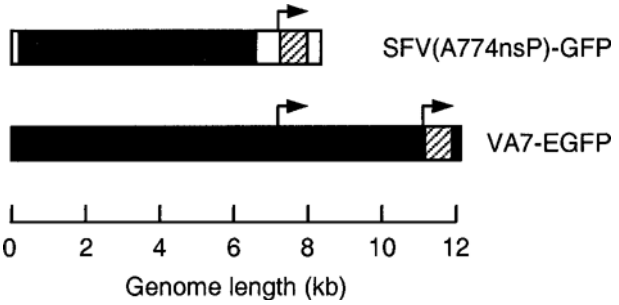

Figure 1 Schematic representation of the viral recombinants used in this study. SFV(A774nsP)-GFP is a replicon composed of *nsP1–4* from A7(74) (*black*) and SFV4 (*white*), VA7-EGFP is a fully replication-proficient virus based on the A7(74) genome (*black*); both viruses carry the reporter gene for enhanced GFP (*hatched*). Arrows indicate the position of the subgenomic promoters.

system is based (Liljeström and Garoff, 1991), we examined whether the GFP expression obtained with the SFV(A774nsP)-GFP replicon and the VA7-EGFP virus is temperature-dependent. We added equal amounts of SFV(A774nsP)-GFP to BHK cells and cultured them at 37℃ and 31℃. At 1 day post infection, only few GFP-positive cells were obtained for SFV(A774nsP)-GFP at 37℃ (Figure 2**A**). Incubation at 31℃, by contrast, resulted in significantly (≈10,000-fold) more GFP-positive BHK cells compared to 37℃ (Figure 2**A**, **B**). Similar data were obtained at 3 days post infection (data not shown). To test whether the viral strain from which SFV(A774nsP)-GFP was derived is also temperature-sensitive, we used VA7-EGFP that contains the full-length A7(74) genome plus the GFP reporter gene. As this virus causes the release of infectious particles from host cells, we restricted our analysis to 1 day post infection. Similar to SFV(A774nsP)-GFP, incubation of BHK cells at 31℃ after addition of VA7-EGFP led to 150-fold more GFP-positive cells compared to 37℃ (Figure 2**A**, **B**). Taken together, these data demonstrate that the changes present in nsP1–4 confer temperature sensitivity to A7(74). This finding might explain the avirulence found for A7(74) upon injection into the CNS of adult mice (Fazakerley *et al*, 1993; Oliver *et al*, 1997) and rats (Sammin *et al*, 1999). We therefore analyzed the pattern of infection in rat hippocampal tissue and cell cultures.

Infection with SFV(A774nsP)-GFP

We injected a total of 28 hippocampal slice cultures from P6 rats with 10- to 1,000-fold diluted SFV(A774nsP)-GFP. Five of them were incubated at 37℃, the remaining 23 slices were incubated at 31℃. As expected from the above experiments in BHK cells, there were significantly more GFP-positive cells at 31℃ than at 37℃ ($206 \approx 11$ versus $78 \approx 26$ cells/slice at 1 day post infection, respectively, $n = 3$ slices per temperature; Figure 3**A**, **D**). When we determined the type of transduced hippocampal cells at the two temperatures, however, a striking phenotype for SFV(A774nsP)-GFP was revealed. Although the SFV4-based vector SFV-GFP transduces mostly neurons at 37℃ (>90% of the GFP-positive cells are pyramidal cells and interneurons; Ehrengruber *et al*, 1999, 2001), SFV(A774nsP)-GFP preferentially transduced glial cells at 37℃ (Figure 3**B**, **C**). A quantitative analysis showed that

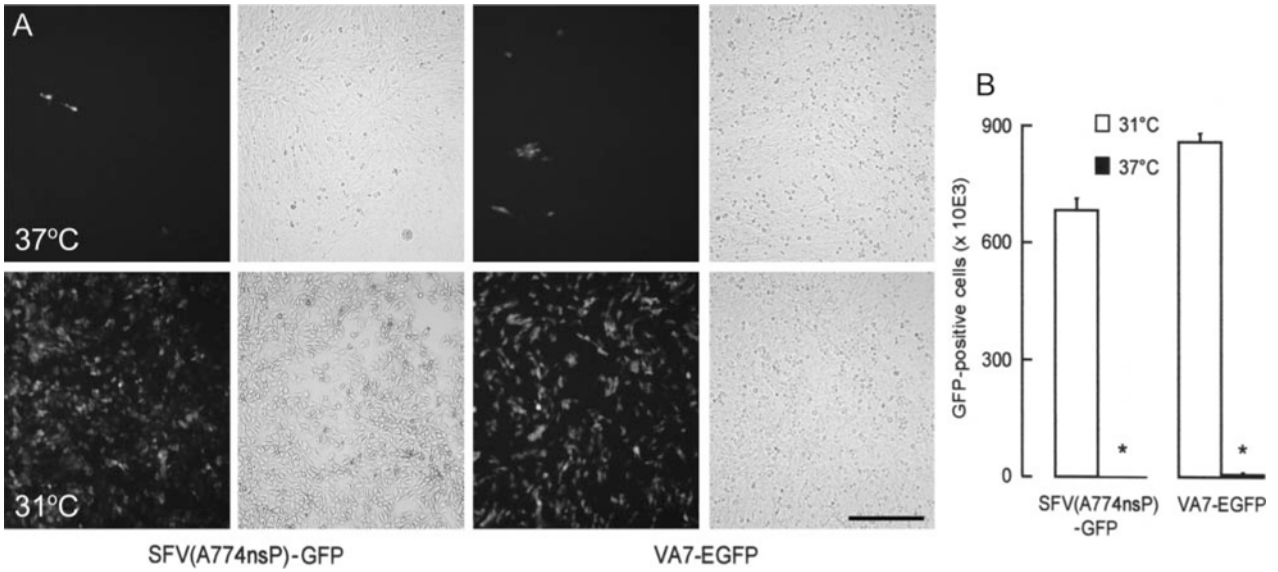

Figure 2 Temperature-dependent GFP expression in infected BHK-21 cells. (**A**) Fluorescence and phase contrast micrographs of living cells at 1 day after infection with the SFV(A774nsP)-GFP replicon (*left*) or the fully replication-competent VA7-EGFP virus (*right*). Directly upon addition of equal amounts of viral stocks (20 and 5 μl, respectively, per dish), cells were incubated at either 37℃ or 31℃ (*top* and *bottom*, respectively). Bar: 160 μm. (**B**) Number of GFP-positive BHK cells per 35-mm plastic Petri dish at 1 day after infection with 20 μl SFV(A774nsP)-GFP or 5 μl VA7-EGFP, and incubation at either 31℃ or 37℃ (means ≈ *SD*, $n = 4$). Asterisks indicate statistically significant differences between the two temperatures tested ($P < .001$, *t* test).

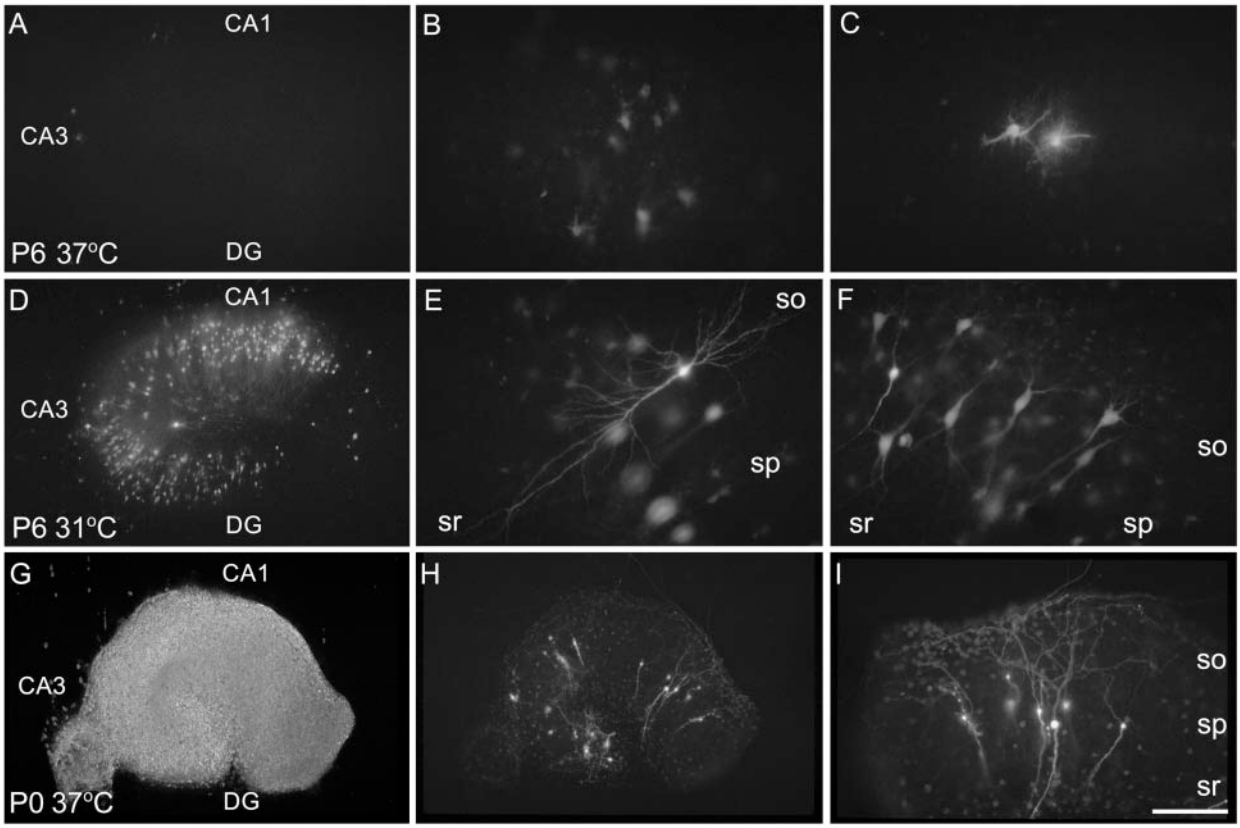

Figure 3 Infection of immature and more mature rat hippocampal slices with SFV(A774nsP)-GFP. Virus was injected at a 100-fold dilution into the pyramidal cell layer from P6 slices of at least 14 days *in vitro* (**A–F**), and into P0 slices at 1 day *in vitro* (**G–I**). Slices were cultured at 37℃ or 31℃ (**A–C** and **G–I** versus **D–F**, respectively), and fixed and analyzed at 1 to 3 or 1 day post infection (P6 versus P0, respectively). Fluorescence micrographs of whole slices (**A**, **D**, **G**, **H**), CA1 region (**B**, **E**, **I**), and CA3 region (**C**, **F**); note that GFP-positive neurons occur at 31℃ but not 37℃ for P6 slices, whereas they are found at 37℃ for P0 slices, and GFP-positive glial cells occur in P6 slices at both 31℃ and 37℃ (c.f., Table 1 for quantification). Similar results were obtained with 10- and 1,000-fold diluted virus. Abbreviations: DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; bar: 300 μm (**A**, **D**, **G**, **H**), 150 μm (**I**), and 75 μm (**B**, **C**, **E**, **F**).

<1% of the GFP-positive cells at 37℃ in hippocampal slices were neurons (Table 1). When the slices were incubated at 31℃, the number of transduced glial cells increased only slightly (from an average of 67 cells/slice at 37℃ to an average of 97 cells/slice at 31℃). However, there was a significant, ≈250-fold increase in the number of GFP-positive neurons (from an average of 0.2 neurons/slice at 37℃ to an average of 48 neurons/slice at 31℃; Table 1), which consisted of CA1 and CA3 pyramidal neurons (Figure 3**E**, **F**) and interneurons, amounting to 27% and 7%, respectively, of all infected cells

Table 1 GFP-positive cell types in rat hippocampal slices infected with wild-type SFV-GFP, SFV(A774nsP)-GFP, and VA7-EGFP

	Slices	*Pyramidal cells*		*Interneurons*		*Non-neuronal cells*		*Total*	
Virus, temperature	n	n	%	n	%	n	%	n	X^2
SFV, 37℃	14	1462	87.4	104	6.2	107	6.4	1673	
SFV(A774nsP), 37℃	5	1	0.3	0	0	333	99.7	334	
SFV(A774nsP), 31℃	12	468	26.9	114	6.5	1160	66.6	1742	152≈
SFV(A774nsP), 37℃, P0	7	62	39.0	12	7.4	89	54.6	163	174≈
VA7-EGFP, 37℃	10	53	3.1	51	2.9	1635	94.0	1739	
VA7-EGFP, 31℃	8	1197	55.4	834	38.6	129	6.0	2160	3015≈

Note. If not otherwise mentioned, P6 slices cultured for at least 14 days were used. Cell types were identified by their characteristic morphology and location within the slice at 1 to 2 days post infection. Glial cells, ependymal cells, and unidentified cells were grouped into non-neuronal cells. The values for SFV-GFP were taken from Ehrengruber *et al* (2001). Chi-square tests comparing the cell type distributions for SFV(A774nsP)-GFP and VA7-EGFP at 31℃ to the ones at 37℃ revealed highly significant differences ($P < .001$) as indicated by the asterisks (the respective X^2 values are given in the last column). A chi-square test also revealed a highly significant difference for SFV(A774nsP) transduction of P0 versus P6 cultures at 37℃ ($P < .001$).

(Table 1). A temperature drop by 6°C thus reverts SFV(A774nsP)-GFP to a more wild-type phenotype. This temperature-dependent change in phenotype did not depend on the age of older organotypic slices prepared from P6 rats, as similar results were obtained for P6 slices at 22, 29, and 42 days *in vitro* (data not shown). We have previously described a similar change back to wild-type phenotype at lower temperature for a different SFV mutant that at 37°C preferentially transduced interneurons in P6 slice cultures (Lundstrom *et al*, 2001). In any case, our present results show that at 37°C, i.e., around body temperature, SFV(A774nsP)-GFP transduces glial cells rather than neurons in more mature hippocampal tissue. The transduction is the result of efficient replication and expression of the viral RNA.

In contrast to adult animals, neonatal rodents are susceptible to A7(74) infection and support the spread of A7(74) in the CNS (Fazakerley *et al*, 1993; Oliver *et al*, 1997). As a control, we thus injected SFV(A774nsP)-GFP into immature hippocampal slices that had been prepared from neonatal (P0) rats and cultured for only 1 day before the virus application (Figure 3**G–I**). When such slices were incubated at 37°C for 1 additional day, prominent GFP expression was observed in both neurons and non-neuronal cells (Figure 3**H**, **I**). As compared to the older P6 slices at 37°C, the number of GFP-positive pyramidal cells and interneurons was significantly increased, reaching 39% and 7% of all transduced cells, respectively (Table 1). These data agree with the previous results obtained in mice, showing that A7(74) replication and expression depend on CNS maturity (Fazakerley *et al*, 1993; Oliver *et al*, 1997). In summary, both neuronal age and temperature affect SFV(A774nsP)-GFP replication and expression in a similar manner.

To verify our results obtained in mature P6 slice cultures, we repeated the experiments in dissociated hippocampal cells obtained from P5 rats and cultured for 7 to 19 days. These cells were infected with SFV(A774nsP)-GFP at 100- to 10,000-fold dilution, incubated at either 37°C or 31°C, and analyzed after 1 day. Upon incubation at 37°C, again, mostly GFP-positive glial cells such as oligodendrocytes were found (Figure 4**A**, *top*), and only 1.2% of all GFP-positive cells were neurons (i.e., 2 out of 165 transduced cells; Figure 4**B**). When infected cultures were incubated at 31°C, by contrast, most of the GFP-positive cells were neurons (i.e., 193 out of 205 transduced cells; Figure 4**A**, *middle*; Figure 4**B**) such as CA1 pyramidal cells (Figure 4**A**, *bottom*), whereas GFP-positive non-neuronal cells were weakly fluorescent and constituted only 6.2% of all GFP-positive cells (12 out of 205 transduced cells; Figure 4**B**). These results thus confirm our data obtained with SFV(A774nsP)-GFP in hippocampal slices. Taken together, our findings

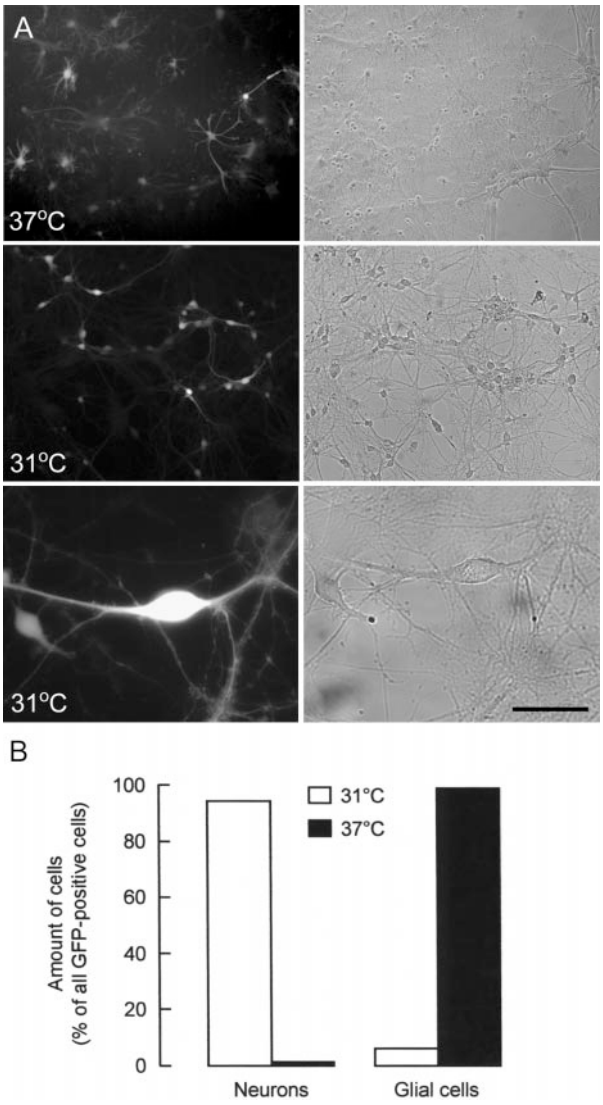

Figure 4 Infection of dissociated P5 rat hippocampal cells with SFV(A774nsP)-GFP. Primary hippocampal cells from 5-day-old rats were infected with 100-fold diluted virus and then cultured at 37°C versus 31°C (*top* versus *middle* and *bottom*, respectively). (**A**) Fluorescence micrographs (*left*) and bright field images (*right*) were taken from living cells at 1 day post infection. Similar results were obtained with 1,000- and 10,000-fold diluted virus. Note that GFP-positive neurons are detected at 31°C rather than 37°C. Bar: 200 μm (*top* and *middle*), and 50 μm (*bottom*). (**B**) Percentage of GFP-positive hippocampal neurons and glial cells out of the total number of GFP-positive cells found at 1 day after incubation at 31°C and 37°C (totals of 205 and 165 analyzed cells, respectively). Note that glial cells are only transduced at 37°C, whereas neurons are transduced at 31°C rather than 37°C.

suggest that the parental A7(74) strain transduces glial cells rather than neurons in the hippocampus at 37°C. In agreement with this hypothesis, previous studies employing replication-proficient A7(74) demonstrated efficient infection of glial cells in dissociated rat CNS cells (Bruce *et al*, 1984; Atkins *et al*, 1990).

Electrophysiological and immunochemical identification of the infected cell types in slices

To confirm the identity of the GFP-positive cells, we performed electrophysiological recordings from hippocampal slices that had been infected with SFV(A774nsP)-GFP and cultured for 1 to 2 days at either 31℃ or 37℃. By overlaying the differential interference contrast and fluorescence images as illustrated previously (Ehrengruber *et al*, 1999), single infected (GFP-positive) cells were easily identified in the pyramidal cell layer for patch clamp analysis. Hyperpolarizing and depolarizing current pulses were then injected to elicit membrane potential changes as previously described (Ehrengruber *et al*, 2001). During depolarization, GFP-positive cells from the pyramidal cell layer of slices incubated at 31℃ generated action potentials, identifying them as neurons (Figure 5**A**, *top*). Their resting membrane potentials (E_m) and conductances (g_m) did not differ from the ones of uninfected CA1 pyramidal cells from the same slices (average E_m of ≈60.9 ≈ 2.4 mV [7] versus ≈59.6 ≈ 1.5 mV [3], and g_m of 8.9 ≈ 1.9 nS (7) versus 8.3 ≈ 1.2 nS [3], respectively; means ≈ SD [*n* cells]). Also, the initial firing was similar to the one in uninfected neurons, as determined by the threshold current I_{Th} required to elicit firing and the initial firing frequency (f_i) to injected current relationship (mean $I_{Th} = +43$ versus +37 pA, and 0.17 versus 0.16 Hz/pA for GFP-positive and control cells, respectively; Figure 5**B**, *squares*). In addition, the firing accommodation, i.e., the ratio between initial and late firing frequency remained unchanged (Figure 5**B**; f_i/f_{late} at +350 pA = 2.27 ≈ 0.47 versus 2.16 ≈ 0.57 for seven GFP-positive and two uninfected cells, respectively, $P = .77$, *t* test). In summary, our data prove that a significant fraction of the GFP-positive cells obtained with SFV(A774nsP)-GFP in slices at 31℃ are neurons, and that the infection does not alter the passive and active membrane properties of CA1 pyramidal cells at 1 to 2 days post infection.

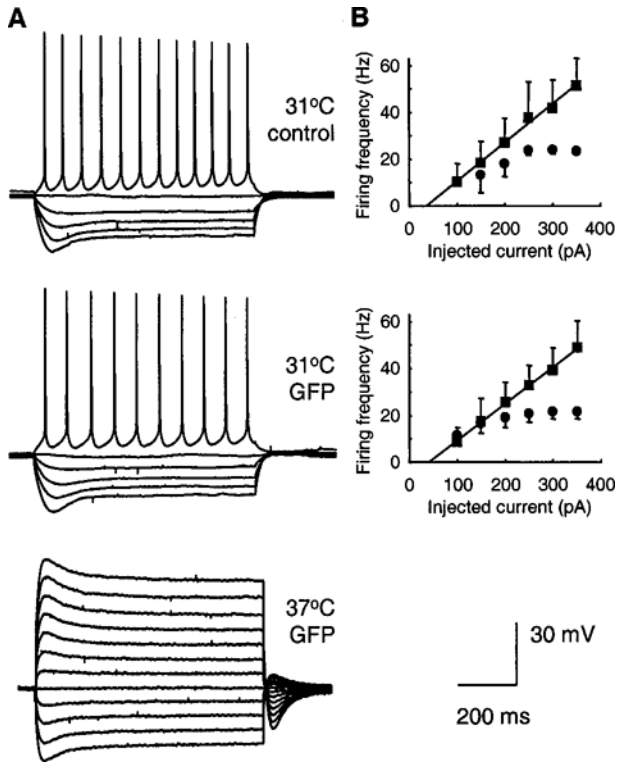

Figure 5 Electrophysiological recordings from uninfected and infected cells of the CA1 pyramidal cell layer from slices treated with SFV(A774nsP)-GFP and incubated at 31℃ or 37℃. (**A**) E_m responses to 0.6-s current pulses (steps of 50 pA) of noninfected (control) and GFP-positive cells at 2 days post infection. Control cells were GFP-negative CA1 pyramidal cells from slices where virus had been applied. Note that GFP-positive cells obtained at 31℃ rather than 37℃ fire action potentials, which identifies them as neurons, whereas the E_m changes of GFP-positive cells obtained at 37℃ are typical of glial cells (Barres *et al*, 1990). (**B**) Means ≈ *SD* (3 control and 7 infected neurons in *top* and *bottom* panels, respectively) of the initial spike frequencies (f_i, squares) and average spike frequencies at 0.4 to 0.6 s (f_{late}, circles) during depolarizing current pulses. Solid lines show the linear fits for f_i ($R^2 = .993$ and .997 for uninfected and infected cells, respectively) with *x*-axis intercepts indicating the rheobase I_{Th} (Ehrengruber *et al*, 1997).

In contrast to cells from slices incubated at 31℃, none of the tested GFP-positive cells of the pyramidal cell layer from slices cultured at 37℃ generated action potentials upon depolarization (Figure 5**A**, *bottom*). In addition, their resting E_m was significantly more negative (≈88.7 ≈ 0.7 mV, three cells) than for the GFP-positive cells obtained at 31℃ (c.f., above; $P < .001$, *t* test), and both depolarizing and hyperpolarizing current pulses caused more symmetrical E_m deflections, which indicates the presence of little voltage-dependent channels. Moreover, prominent tail currents occurred after the 0.6-s current pulses. All these electrophysiological data are characteristic of glial cells (Barres *et al*, 1990) and, taken together, strongly suggest that the GFP-positive cells obtained in slices with SFV(A774nsP)-GFP at 37℃ are non-neuronal.

To further examine the cell types infected by SFV(A774nsP)-GFP, we performed triple immunostaining in a total of 20 hippocampal slice cultures by using primary antibodies that recognized markers for mature astrocytes (glial fibrillary acidic protein, GFAP), neurons (neuronal nuclear antigen, NeuN), and oligodendrocytes (RIP) (Ghandour *et al*, 1979; Friedman *et al*, 1989; Mullen *et al*, 1992). When the infected slices were incubated at 37℃, no NeuN/GFP double-positive cells were observed (Figure 6**A**), which indicates that neurons were not efficiently transduced at this temperature and agrees with our morphological and electrophysiological analyses. By contrast, virtually all GFP-positive cells (20 to 40 cells per slice) were immunoreactive for GFAP (Figures 6**A**, 7) but not for RIP (Figure 7), suggesting a strong preference for transduction of astrocytes rather than oligodendrocytes. Under our culture conditions, RIP staining was generally sparse (<30 cells per slice; Figure 7**A**), which may reflect an altered development of this cell population in

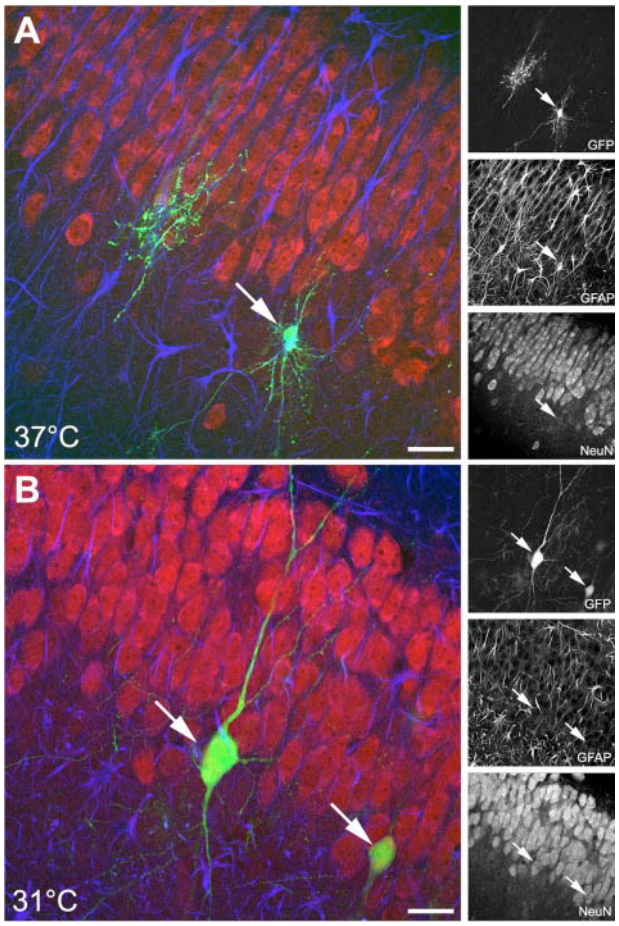

Figure 6 Confocal microscopic analysis of the phenotype of transduced cells from slices infected with SFV(A774nsP)-GFP and incubated at 37℃ and 31℃ (*top* and *bottom*, respectively). (**A**) After incubation at 37℃, GFP-expressing cells (*green*, *arrow*) have small cell bodies that are positive for GFAP (*blue*) but not NeuN (*red*), identifying them as astrocytes. (**B**) Upon incubation 31℃, infected cells (*green*, *arrows*) were frequently detected in the pyramidal cell layer, had large cell bodies, a typical polarized morphology, and were strongly immunoeractive for NeuN (*red*), which identifies them as pyramidal cells. Scale bars: 100 μm (**A**) and 50 μm (**B**).

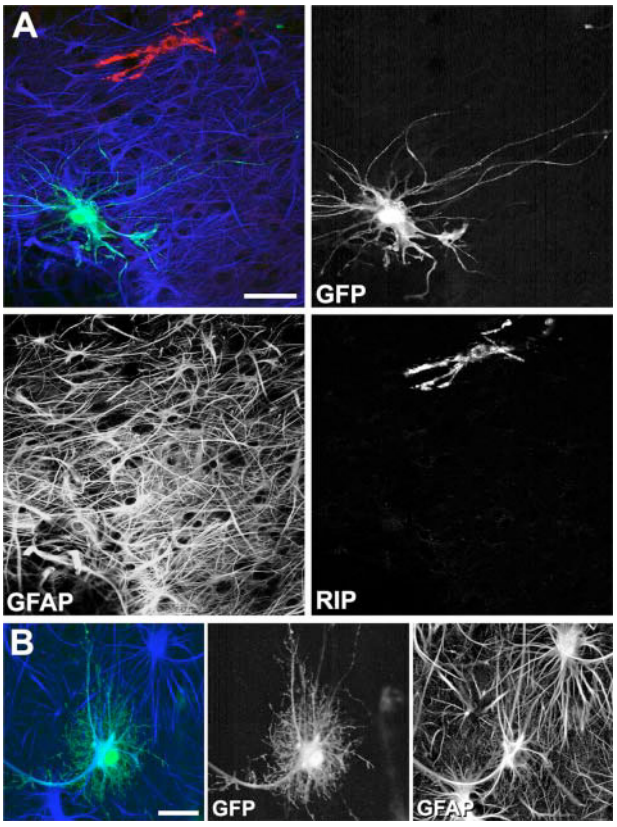

Figure 7 At 37℃, SFV(A774nsP)-GFP transduces astrocytes rather than oligodendrocytes in organotypic hippocampal slices. (**A**) Representative confocal microscopic image showing a triple immunostaining. The GFP expression of an infected cell (*green*) colocalizes clearly with GFAP immunoreactivity (*blue*) but not RIP immunoreactivity (*red*), which identifies infected glial cells as astrocytes but not oligodendrocytes. (**B**) A GFP/GFAP double immunoreactive cell of the astroglial scaffold at the surface of a slice. Scale bars: 50 μm (**A**, **B**).

organotypic cultures and/or the high vulnerability of this cell type to the irradiation and antimitotic treatment used herein (Berger and Frotscher, 1994). In addition, few RIP-positive cells had the profile of myelinating oligodendrocytes (not shown), and none of these showed any GFP expression. Of the GFP-positive astrocytes, the vast majority had a highly ramified morphology typical of type II astrocytes. They were present both at the surface of the culture (within the well described astroglial scaffold on the organotypic slices [del Rio *et al*, 1991]; Figure 7**B**) and deeper within the slice, where they form radial processes between pyramidal cells (Figure 6**A**).

As opposed to incubation at 37℃, numerous GFP-expressing cells could be identified as neurons when the infected slices were cultured at 31℃. These cells possessed the typical polarized morphology of pyramidal cells, as identified by GFP immunostaining, and expressed the mature neuronal marker NeuN but not the glial marker GFAP (Figure 6**B**). Besides these neurons, astrocytes were also infected at 31℃, in a similar pattern as at 37℃. Taken together, these immunocytochemical data confirm our results based on the morphological analysis of GFP-fluorescent cells (above), showing that SFV(A774nsP) is able to transduce neurons at 31℃ but not 37℃.

Infection with VA7-EGFP

To examine the infection pattern obtained with the parental strain of the SFV(A774nsP)-GFP vector in hippocampal slice cultures, we employed recombinant A7(74) expressing GFP (VA7-EGFP). A total of 29 slices was injected with undiluted or 10- to 10,000-fold diluted VA7-EGFP, incubated at either 37℃ or 31℃, and analyzed at 1 and 2 days post infection. As the virus was fully replication-competent, the number of infected cells was increased at 2 days compared to 1 day post infection (Figure 8**A**, **B**). Infectious

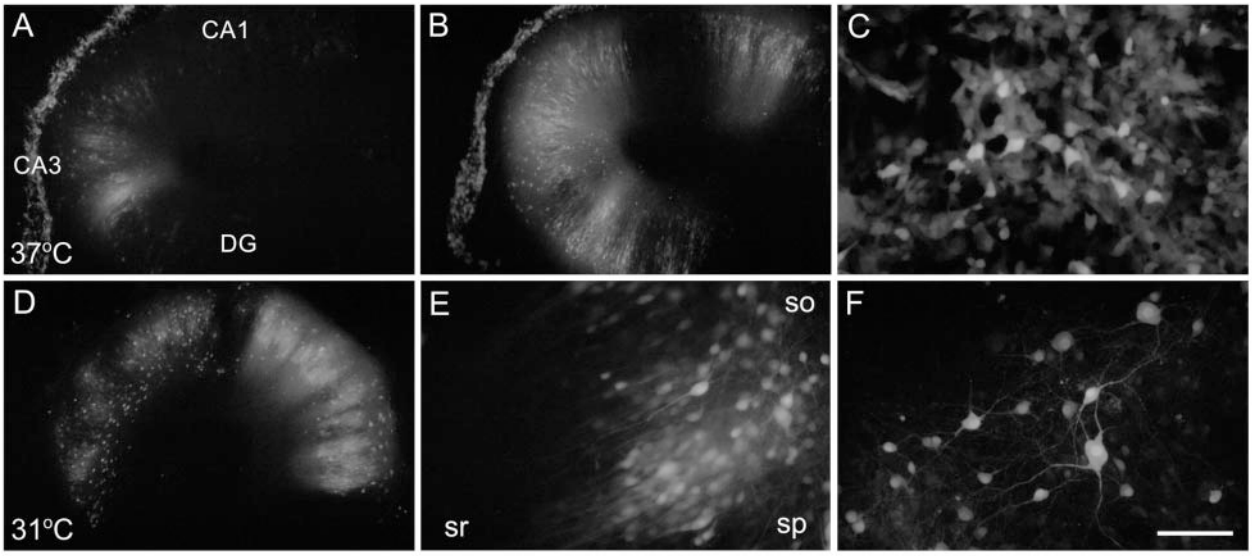

Figure 8 Infection of rat hippocampal slices with VA7-EGFP. Upon virus application, slices were cultured at 37℃ or 31℃ (**A–C** and **D–F**, respectively) and fixed after 2 days. Fluorescence micrographs of whole slices at 1 and 2 days post infection (**A** and **D** versus **B**, respectively; **A** and **B** show the same slice), GFP-positive ependymal cells (**C**), CA1 pyramidal cells (**E**), and tyrosine hydroxylase–positive neurons from the CA1 region (**F**; c.f., Knöpfel *et al*, 1989). Note the prominent GFP staining of non-neuronal, ependymal cells along the perimeter of the slice at 37℃ but not 31℃ (**C** shows a magnification of these cells). Similar results were obtained with undiluted and 10- to 10,000-fold diluted virus. Abbreviations as in Figure 3. Bar: 300 μm (**A**, **B**, **D**), 75 μm (**C**, **E**, **F**).

virus was detected in the medium, demonstrating that VA7-EGFP propagates in hippocampal slices via the release of free, infectious particles from infected cells. No virus was found in medium from slices infected with SFV(A774nsP)-GFP (data not shown).

We next examined the type and number of cells with GFP fluorescence as a function of incubation temperature. At 37℃ and 1 day post infection, ≈95% of the GFP-positive cells from slices at 24 days *in vitro* were non-neuronal cells (Table 1), such as ependymal cells along the perimeter of the slices (Figure 8**A–C**). Slices incubated at 31℃, by contrast, had no GFP-positive ependymal cells. They showed a significantly elevated amount of transduced CA1 and CA3 pyramidal neurons and interneurons, representing 55% and 39%, respectively, of all GFP-positive cells (Table 1, Figure 8**D–F**). When VA7-EGFP infectivity spread with increasing time post infection, the number of GFP-positive pyramidal cells and interneurons was augmented at 2 days post infection, whereas no newly infected non-neuronal cells were observed at both 31℃ and 37℃ (Figure 8**B**). Taken together, our results with VA7-EGFP agree with the data obtained using SFV(A774nsP)-GFP in that viral replication and expression (as deduced from the GFP reporter fluorescence) is limited in neurons at 37℃ but not 31℃. A7(74) appears to be avirulent in adult rodents because, in contrast to virulent SFV4 (from which conventional SFV vectors have been derived), it does not readily replicate in principal CNS neurons at body temperature, which fluctuates between 36.6℃ and 39.4℃ in mice (Woodward and Smith, 1979; Hearne *et al*, 1987).

Discussion

In situ hybridization, immunocytochemistry, and electron-microscopic experiments have shown that mature rat and mouse neurons can be infected by the avirulent SFV strains A7 and A7(74), but most of these neurons remain morphologically normal (Fazakerley *et al*, 1993; Balluz *et al*, 1993; Oliver *et al*, 1997; Sammin *et al*, 1999). In neonatal rodents, by contrast, avirulent SFV causes a widespread, fatal infection (Fleming, 1977; Atkins, 1983; Fazakerley *et al*, 1993; Oliver *et al*, 1997; Oliver and Fazakerley, 1998). It was therefore concluded that the cellular basis for avirulence is the ability of adult neurons to restrict viral multiplication (Gates *et al*, 1985; Fazakerley *et al*, 1993; Oliver *et al*, 1997; Sammin *et al*, 1999). Although it is clear that host cell proteins are involved in alphaviral RNA synthesis (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 2001), the viral and cellular processes leading to restriction of viral replication at the molecular level remain unknown, as is the mechanism of abrogation of this restriction by virulent SFV and SIN strains. We show here that temperature can have a profound effect on restriction of growth: mature neurons can efficiently be transduced by A7(74), albeit at lower temperature (31℃), a temperature that does not easily support A7(74) replication in glial cells, at least in dissociated cultures. In contrast, glial cells are permissive for growth of virus (and replicons) at 37℃ in both organotypic and dissociated cultures. To our knowledge, this is the first report of temperature affecting in a dual manner which cell type can replicate

SFV A7(74). In agreement with our findings, A7 and A7(74) infection and replication in rat and mouse oligodendrocytes and other glial cells at 37℃ has been previously described (Bruce *et al*, 1984; Gates *et al*, 1985; Fazakerley *et al*, 1993; Balluz *et al*, 1993). In addition, a preference for glial cells rather than neurons was noted in both rat and mouse brain cell cultures at 37℃ (Bruce *et al*, 1984; Gates *et al*, 1985).

Besides the temperature, other physical or chemical alterations can affect the replication of A7(74). Scallan and Fazakerley (1999) described increased A7(74) replication and virulence in the CNS of adult mice that had been pretreated with aurothiolates (Scallan and Fazakerley, 1999). These compounds, therefore, also seem to affect some particular factor(s) determining A7(74) avirulence in mature neurons, similar to the temperature in our *in vitro* preparations. The aurothiolate experiments were performed in living animals, where cytokines, T lymphocytes, and a multitude of other factors also may affect the virulence of A7(74). In any case, further experiments will have to test whether the factor(s) influenced by aurothiolates is identical to the one(s) influenced by temperature.

Our comparison of SFV(A774nsP)-GFP and VA7-EGFP in hippocampal slice cultures at 37℃ revealed a less restricted neuronal transduction for VA7-EGFP (c.f., Figure 3 versus Figure 8; Table 1). This effect became more pronounced at 2 days rather than 1 day post infection (c.f., Figure 8**A**, **B**). The different transduction patterns for SFV(A774nsP)-GFP and VA7-EGFP may result from amino acid alterations in the structural proteins, which have been previously shown to affect alphaviral virulence (Davis *et al*, 1986; Lustig *et al*, 1988; Glasgow *et al*, 1994; McKnight *et al*, 1996; Bernard *et al*, 2000). We therefore performed sequence alignments for the structural proteins of pSFV-Helper2 (Genbank accession number AR130391; Berglund *et al*, 1993), which we used to package the SFV(A774nsP)-GFP replicons, and of the VA7-EGFP parental virus A7(74) (Genbank accession numbers X78109, X78110, X78112, X78111, and X74425 for the capsid, E3, E2, 6K, and E1 protein, respectively; Santagati *et al*, 1994, 1995). There was no difference for the 6K protein, but the following amino acids were altered in pSFV-Helper2 as compared to A7(74): T62A and R63G (capsid); T12A and A24V (E3); *S212N*, K215M, A367V, A371V, and A389V (E2); *S65A*, *K115R*, T228M, T297I, and K373R (E1). Thus, although some of these changes are more conservative (in italics), the other amino acid changes, either alone or in combination, may contribute to the slightly different transduction characteristics obtained with SFV(A774nsP)-GFP and VA7-EGFP at 37℃ in hippocampal slices.

Fazakerley *et al* examined the spread of A7(74) through the CNS of mice and found transmission along hippocampal pathways to decrease with age, ceasing by P8, i.e., when hippocampal connectivity is mostly complete (Fazakerley *et al*, 1993; Oliver *et al*, 1997). In this study, we used hippocampal slices prepared from P6 rats and cultured for an additional average of 25 days. By that time, the neuronal connectivity in this system has matured (McKinney *et al*, 1999). Similarly, the cultures of dissociated hippocampal cells were prepared from P5 rats and cultured for another 7 to 26 days. We thus expect our *in vitro* systems derived from P5 and P6 rats to represent a more adult rather than neonatal state of cellular physiology. It has been suggested for SIN that fatal infection of P4 and younger mice occurs by shock like stress, with little inflammation but high levels of endotoxic cytokines, whereas nonfatal infection in P8 mice is characterized by extensive inflammation and encephalitis (Trgovcich *et al*, 1999). Inflammation is normally paralleled by fever. One could thus speculate that A7(74) is neurovirulent in neonatal rodents as their body temperature might be lower than in adult animals, where a temperature rise during fever would additionally restrict A7(74) replication in neurons. For A7 infection in adult mice, however, an increase in body temperature did not occur (Woodward and Smith, 1979).

In contrast to virulent forms, A7(74) and avirulent SIN do not cause apoptosis in the CNS of older animals, but they do so in neonatal mice (Lewis *et al*, 1996; Fazakerley *et al*, 2002). In agreement with this, no evidence for A7-induced apoptosis was found in the CNS of mature rats (Sammin *et al*, 1999). Similarly, A7-infected rat neurons *in vitro* do not undergo typical apoptosis but rather necrosis (Glasgow *et al*, 1997). It was thus hypothesized that developmental changes occur in the neuronal susceptibility towards alphavirus-induced apoptosis, with avirulent SFV and SIN causing apoptotic death in immature neurons, but necrosis in terminally differentiated neurons (Lewis *et al*, 1996; Sammin *et al*, 1999; Allsopp and Fazakerley, 2000).

Two possible modes have been proposed for the switch from virulence to avirulence of A7(74) in neonatal versus adult rodents: (1) a developmental change in expression of the virus receptor molecule on neurons, and (2) the ability of neonatal, but not adult neurons, to replicate virus (Oliver *et al*, 1997). The data presented herein seem to exclude the first alternative and favor the second explanation, as our A7(74) recombinants were able to replicate and express in hippocampal neurons at lower temperature. In agreement with a reduced ability of mature hippocampal neurons to replicate A7(74) at 37℃, it was shown that primary rat neuronal cultures produce at 37℃ less RNA for A7 and A7(74) than for virulent SFV (Atkins, 1983; Atkins *et al*, 1990; Tuittila *et al*, 2000). In general, virulence appears to relate to the rate of SFV multiplication in the CNS, resulting in a lethal threshold of damage before immune intervention can occur; this threshold is not reached by avirulent SFV, and immune responses result in survival (Balluz *et al*, 1993). The restriction of A7 and A7(74) replication in neurons by itself is

not immunologically determined and occurs in both immune-deficient and -competent mice (Fazakerley *et al*, 1993, 2002; Amor *et al*, 1996).

Our results on the temperature-sensitivity of the A7(74)-based recombinants were not unexpected as earlier reports have described temperature-sensitive RNA synthesis and growth for A7 in BHK cells and brain tissue cultures (Woodward and Smith, 1979; Hearne *et al*, 1987). Although one study describes an ≈twofold lower growth and approx. 23% lower viral RNA synthesis at 37℃ compared to 30℃ (Hearne *et al*, 1987), we found a more dramatic change in GFP expression for SFV(A774nsP)GFP and VA7-EGFP at 37℃ versus 31℃ (c.f., Figure 1). A chemically induced, temperature-sensitive A7 mutant (*ts4*) described by Atkins and colleagues, however, did show 10,000-fold lower viral growth at 37℃ compared to 30℃ in BHK cells. Similarly, strong temperature sensitivities have been obtained with specific mutant SFV and SIN vectors (Boorsma *et al*, 2000; Lundstrom *et al*, 2001). Both *in vitro* and *in vivo*, A7 and A7(74) infect and replicate in oligodendrocytes and specific astrocytes, causing cytopathic effects and depletion of these cells (Bruce *et al*, 1984; Gates *et al*, 1985; Atkins *et al*, 1990; Fazakerley *et al*, 1993; Balluz *et al*, 1993; Glasgow *et al*, 1997). The glial preference, which in our slice culture system was restricted to astrocytes, could explain why at body temperature A7(74) is avirulent. It would be interesting to isolate and identify the temperature-sensitive host cell factor(s) permitting A7(74) replication in glial cells at 37℃ and in neurons at 31℃.

Materials and methods

Cell and tissue cultures

BHK cells were grown at 5% CO_2/95% air and at 37℃ in Dulbecco's modified Eagles medium containing 4.5 g/L glucose (Invitrogen), 10% fetal bovine serum (Invitrogen), 4 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Postnatal day 5 (P5) rat hippocampal cells were cultured on glass cover slips at 37℃ until infection in 35-mm Petri dishes as described (Malgaroli *et al*, 1995). Hippocampal slices from neonatal (P0) and P6 rats were cultured at 37℃ in the roller-tube configuration according to the standardized protocol (Gähwiler, 1981).

Generation of recombinant virus particles

The 6418 bp long *EcoR* V/*Bgl* II fragment encompassing *nsP1–4* (except for the 5′-terminal 192 nucleotides of *nsP1* and the 3′-terminal 585 nucleotides of *nsP4*) of the A7(74) cDNA (Tuittila *et al*, 2000) was subcloned into the corresponding restriction sites of the pSFV-GFP replicon (Ehrengruber *et al*, 1999), which is based on SFV4, to generate pSFV(A774nsP)-GFP. The construction of the replication-proficient A7(74) vector, VA7-EGFP, expressing enhanced GFP under a second subgenomic promoter in the 3′-nontranslated region is described elsewhere (M. J. V. Vähä-Koskela, M. T. Tuittila, P. T. Nygårdas, J. K.-E. Nyman, M. U. Ehrengruber, M. Renggli, and A. E. Hinkkanen, 2003).

To produce viral particles, RNA from *in vitro* transcribed pSFV(A774nsP)-GFP was introduced with RNA from pSFV-Helper2 (Berglund *et al*, 1993) into BHK cells by electroporation. RNA for VA7-EGFP was transfected into BHK cells by itself, and all electroporated cells were incubated at 31℃. Recombinant SFV particles were harvested after 24 to 48 h, and, for SFV(A774nsP)-GFP, activated with alpha-chymotrypsin (Berglund *et al*, 1993). They had a titer of $\approx 10^8$ infectious particles/ml (as determined on BHK cells).

Infection and analysis of GFP expression

BHK cells in 35-mm Petri dishes and dissociated hippocampal cells at 7 to 19 days in culture were infected by adding virus at a 100- to 10,000-fold dilution to the culture medium. Slice cultures (for P6 slices at 25.2 ≈ 8.7 days *in vitro*, mean ≈ SD, $n = 6$ batches; 5–15 cultures/batch) were injected with ≈0.5 μl virus over 10 to 15 sites in both the CA1 and CA3 region as previously described and illustrated (Ehrengruber *et al*, 2002; Ehrengruber and Lundstrom, 2002). Briefly, the infections were performed at room temperature under biosafety level 2 containment by microinjection of virus into the extracellular space of the slice cultures. The tip of autoclaved micropipettes, pulled from borosilicate glass (Clark Electronical Instruments, Reading, England), was broken to a diameter of ≈20 μm. Pipettes were filled with viral solution and, by using a micromanipulator (Narishige, East Meadow, NY), placed into either the CA1 or CA3 pyramidal cell layer. For each placement, one short injection (<2 s) was performed by applying pressure from a 1-ml syringe. Upon infection, cells and slices were incubated at either 31℃ or 37℃ (as indicated).

Hippocampal slices were fixed with 4% (*w*/*v*) paraformaldehyde (Fluka, Buchs, Switzerland) in 0.1 M phosphate buffer (PB) for 4 to 12 h at 4℃ in the dark, washed with PB, and bathed in PB for examination. Living slices were analyzed in their roller tubes on an inverted Axiovert 100 microscope (Zeiss, Feldbach, Switzerland) at 100-fold magnification for cell counting. GFP fluorescence was excited with fluorescein isothiocyanate (FITC) filter sets (Zeiss), and photographs of cell and tissue cultures were taken with an upright Axioplan microscope (Zeiss). Pyramidal neurons, interneurons, and glial cells were identified based on their characteristic morphology and, in the case of tissue cultures, location within the slices.

All data are expressed as the mean ≈ *SD*. For the statistical examination of the number of GFP-positive cells per slice and infected cell types, Student's *t* and chi-square tests were applied, respectively.

Electrophysiology

Whole-cell patch-clamp recordings in hippocampal slice cultures were performed as described (Ehrengruber *et al*, 2001), but using an intracellular solution containing 140 mM potassium gluconate, 10 mM KCl, 5 mM Hepes, 4 mM $MgCl_2$, 1 mM EGTA, and 10 mM phosphocreatine (pH 7.3 adjusted with gluconic acid; 280–290 mOsm). A liquid junction potential of ≈7.1 mV measured as described (Neher, 1992) was taken into account for all data. Only cells with a resting E_m of <≈50 mV were analyzed; g_m was determined by applying a ≈50 pA current step, and E_m responses to 0.6-s current pulses (steps of 50 pA) were recorded. Data are expressed as the mean ≈ *SD*.

Immunofluorescence

Organotypic cultures were processed for multiple markers to determine the cellular phenotype of the infected cells. Primary antibodies were chosen that recognize mature astrocytes (GFAP), neurons (NeuN), and oligodendrocytes (RIP) (Ghandour *et al*, 1979; Friedman *et al*, 1989; Mullen *et al*, 1992).

Organotypic slices were fixed overnight at 4℃ in 4% paraformaldehyde in 0.1 M PB. They were then removed from their support, and the immunostaining procedure was performed in freely floating slices. After extensive washing in 0.1 M PB, blocking and permeabilization were performed in 0.1 M PB containing 0.4% (*v*/*v*) Triton X-100 and 5% (*v*/*v*) normal horse serum for 12 h at 4℃. Three compatible primary antibodies were applied together for 2 days at 4℃ in 0.1 M phosphate-buffered saline (PBS) supplemented with 0.4% (*v*/*v*) Triton X-100 and 2.5% (*v*/*v*) normal horse serum. The following antibodies were used at the given dilutions: mouse anti-NeuN (1:400; Chemicon, CA, USA), guinea pig anti-GFAP (1:250; Advance Immunochemical, CA, USA), rabbit anti-GFP (1:250; Molecular Probes, OR, USA), mouse anti-RIP (1:100; Developmental Studies Hybridoma Bank, IA, USA). Cultures were rinsed four times in 0.1 M PB containing 0.4% (*v*/*v*) Triton X-100 for 30 min, each, before application of the secondary antibodies. The following corresponding secondary antibodies were applied overnight at 4℃ in 0.1 M PBS, 0.4% (*v*/*v*) Triton X-100, and 2.5% (*v*/*v*) normal horse serum, at a dilution of 1:250, each: donkey anti-mouse Alexa 546, donkey anti-rabbit Alexa 488, and donkey anti-guinea pig Alexa 660 (all from Molecular Probes, OR, USA). The signal of the mouse anti-RIP antibody was enhanced by using a biotinylated secondary antibody (horse anti-mouse antibody; Vector Laboratories, CA, USA) followed by a 45-min incubation with streptavidin conjugated to Cy3 (1:250 dilution; Jackson Immunoresearch, PA, USA).

After extensive washings, the cultures were mounted with the ProLong Antifade Kit (Molecular Probes, OR, USA) and kept in the dark at 4℃ until analysis. For the examination, a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Switzerland) with a 40≈ lens (1.25 N.A.) was used.

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