

Cerebral Metabolism in Brains of Rats Infected with Neuropathogenic Murine Leukemia Viruses

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ABSTRACT. Friend murine leukemia virus A8 and PVC211 cause spongiform neurodegeneration in rat brains. Glutamate is an important neurotransmitter synthesized from α -ketoglutaric acid, an intermediate product of the citric acid cycle, and glutamine is synthesized from glutamate. To examine the brain metabolism of rats infected with neuropathogenic viruses, the amount of glutamate and glutamine in the brains of rats infected with A8, PVC211, and non-neuropathogenic 57 was measured using high performance liquid chromatography, and the ¹³C-label incorporation into the C4 position of glutamate and glutamine from [1-¹³C] glucose was measured with ¹³C nuclear magnetic resonance. In the cerebral hemisphere and region containing the brain stem and basal ganglia of rats infected with A8 and PVC211 at 8–9 weeks post-infection (wpi), the amount of glutamine was decreased compared with the 57-infected rats. The amount of glutamate was decreased in the cerebral hemisphere of the A8-infected rats and the region containing the brain stem and basal ganglia of PVC211-infected rats at 8–9 wpi. The amount of [4-¹³C] glutamine and [4-¹³C] glutamate in the cerebral hemisphere and region containing the brain stem and basal ganglia of rats infected with A8 and PVC211 at 8–9 wpi was equivalent to that of the 57-infected rats. These results suggest that in the brains of rats infected with neuropathogenic viruses, *de novo* synthesis of glutamate and glutamine is not decreased, but the ability to maintain quantitative levels of glutamate and glutamine is decreased compared with the brains of rats infected with non-neuropathogenic virus.

KEY WORDS: cerebral metabolism, ¹³C, mouse retrovirus, neuropathogenicity.

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Murine leukemia viruses (MLVs) cause neurodegenerative diseases without inflammatory infiltrates. The first case that showed that murine retroviruses could induce neurological disorders was found in wild mice, which developed hind limb paralysis accompanied with replication-competent ecotropic MLVs, from the Lake Casitas region of California [9, 16]. Ecotropic CasBrE MLV isolated from these mice caused a similar disease in the central nervous system (CNS) in laboratory mice [7]. Since the discovery of CasBrE MLV 20 years ago, other neuropathogenic murine retroviruses have been found. In the ecotropic host range group, variants of Friend MLV, including PVC211 [10], NT40 [4], A8 [25], and PVC441 [27], and temperature sensitive-mutants of Moloney MLV, including ts1 [30, 31] and ts-BA1 [2], cause neurodegenerative disease in mice and/or rats. The neuropathology of these viruses is characterized by spongiform neurodegeneration without inflammatory infiltrates, primarily involving the motor system of the brain and spinal cord, and is associated with widespread astrogliosis and neuropil vacuolation [1, 3, 17, 24]. One common feature of these viruses, including A8 and PVC211, is that the primary determinant for induction of neurodegenerative disease is the *env* gene, although other viral genes also have an effect on neuropathogenicity [5, 6, 15, 20, 21, 26, 28]. Some uninfected neurons may exhibit cytopathogenicity, indicating an indirect mechanism of MLV-induced neuropathogenicity [8, 19]. However, the pathomechanism of

spongiosis induced by *env* and other genes is still not understood.

¹³C Nuclear magnetic resonance (NMR) technology has been used to study the oxidative metabolism of glucose because the amount of ¹³C label in each carbon position within a compound can be quantified [11, 13, 22, 23]. As shown in Fig. 1, after administration of [1-¹³C] glucose into the animals, the [1-¹³C] glucose incorporated into brain cells is converted to [3-¹³C] pyruvate and non-labeled pyruvate by glycolysis. This pyruvate is converted to acetyl-CoA through the pyruvate dehydrogenase complex and then enters the citric acid cycle. In the brain, [3-¹³C] pyruvate labels the C4 position of glutamate in the first turn of the citric acid cycle through [4-¹³C] α -ketoglutaric acid. Therefore, the rate of ¹³C incorporation into glutamate reflects the rate of uptake of glucose and glycolysis, and the citric acid cycle activity. Subsequently, [4-¹³C] glutamate is converted into [4-¹³C] glutamine by the astrocyte-specific enzyme, glutamine synthetase.

In this study, we examined cerebral metabolism of rats infected with neuropathogenic viruses, A8 and PVC211, and non-neuropathogenic 57. A8 and PVC211 are closely related but molecularly distinct. The A8 virus shares a high degree of homology with PVC211 in LTR (86.2%), *gag* (99.6%), *pol* (99.5%), and *env* (99.7%) [25]. The A8 virus induces marked spongiform degeneration in the cortex, thalamus, cerebellum, and brain stem similar to PVC211, but only mild lesions are found in the spinal cord compared with those after PVC211 infection [25]. The 57 virus is a non-neuropathogenic MLV [18]. When 57 is inoculated into

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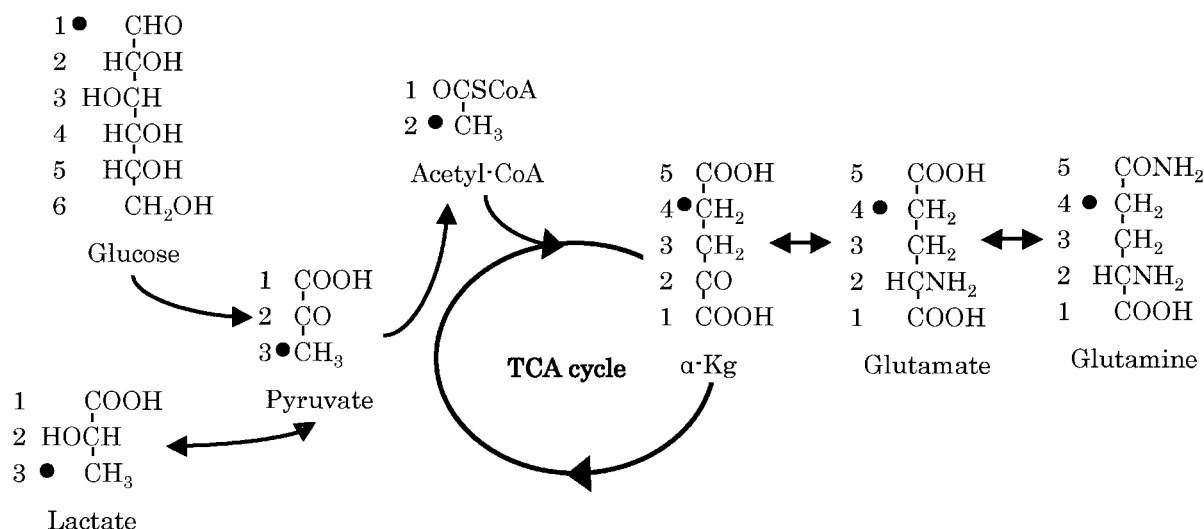


Fig. 1. ^{13}C -labeling to carbon atoms in cerebral metabolites after administration of $[1-^{13}\text{C}]$ glucose. Closed circles represent carbon atoms labeled with ^{13}C in glycolysis and the first turn of the citric acid cycle.

rats, the virus is recovered from the brains of infected rats [25]. There is high degree of homology between 57 and A8 in LTR (91.7%), *gag* (97.7%), *pol* (97.7%), and *env* (99.5%). The results of metabolic analyses suggest that in the brains of rats infected with neuropathogenic viruses, *de novo* synthesis of glutamate and glutamine is not decreased compared with that in the brains of rats infected with non-neuropathogenic virus, but the ability to maintain quantitative levels of glutamate and glutamine is decreased. Furthermore, we discussed the relationship between cerebral metabolism and neuropathogenicity.

MATERIALS AND METHODS

Virus infection: Neuropathogenic FrC6 virus, clone A8, was obtained as described previously [25, 29]. The infectious DNA of non-neuropathogenic clone 57 of Friend MLV [18] and A8 was transfected into NIH3T3 cells, and a virus-producing cell culture was established. The supernatants of these cells were used to infect NIH3T3 cells, and virus-producing cultures were used in the experiments. PVC211-producing normal rat kidney cells were kindly provided by Dr. K. Kai (Yamaguchi University, Yamaguchi, Japan). The ability of viruses to cause disease was assessed using newborn Lewis rats purchased from a commercial breeder. Newborn rats were inoculated intraperitoneally with 0.1 ml and intracerebrally with 0.005 ml of viral supernatant as described previously [25]. Viral titers in the brains were measured by XC plaque assay as described previously [25].

Animal preparation for cerebral metabolic experiment by ^{13}C NMR: $[1-^{13}\text{C}]$ Glucose (99 atom %) and deuterium oxide (99.9 atom %) were purchased from ISOTEC (Miamisburg, OH, U.S.A.). The rats infected with the neuropathogenic viruses were fasted for 20-hr prior to the cere-

bral metabolic experiments. These rats were injected intravenously with a bolus of $[1-^{13}\text{C}]$ glucose (1 g/kg) via the tail vein. Then, the rats were sacrificed by exposure to microwaves for 1.2 s at 4.8 kW (Microwave Applicator; TMW-6402C, Muromachi Kikai, Tokyo, Japan) 15 min after glucose administration. The brains were removed and homogenized in 75% ethanol using an ultrasonic homogenizer. The homogenates were centrifuged at 3000 r.p.m. for 15 min at 4°C. Aqueous extracts were prepared by the addition of water-saturated chloroform to the supernatants and vacuum-dried using a Speed Vac SVC100 (Savant Instruments, Farmingdale, NY).

NMR analysis: The dried samples were dissolved in deuterium oxide containing 0.1 M phosphate buffer (pH 7.4), and placed in an NMR tube (5 mm diameter; PS-003, Shigemi, Hachioji, Japan). The ^{13}C signals from the metabolites were measured using ^1H -decoupled ^{13}C NMR spectroscopy (EX-400, 9.4 T, JEOL, Tokyo, Japan) as described previously [11]. Spectra were acquired under the following conditions: pulse width, 45°; spectral width, 30,120 Hz; acquisition time, 1.09 s; time-domain points, 64,000; pulse interval, 8s; scans, 4000. As an external standard, 29 mM of sodium-3-(trimethylsilyl)-propionate-2,2,3,3- d_4 (TSP) was used per sample. The sample volume was 500 μl . The total amount of ^{13}C was calculated by integrating the peaks obtained in the ^{13}C NMR spectra and using TSP as an external standard. Corrections were made for saturation and differences in Nuclear Overhauser Enhancement. The percent ^{13}C enrichment of the C4 positions of the metabolites was determined as follows: the amount of ^{13}C incorporated in C4-glutamate or C4-glutamine normalized by the amount of ^{13}C of 29 mM TSP/the total amount of the amino acid.

Measurement of amino acid contents by HPLC: The amino acid contents of the NMR samples were measured by

HPLC (high performance liquid chromatography) using the Pico-Tag method with 5 μ l of the aqueous extract after vacuum drying. The assay conditions were as follows: HPLC system, Waters 600F pump, 600E system controller, 484 tunable absorbance detector and 717 autosampler; column, Pico-Tag column (3.9 mm \times 30 mm); column temperature, 46°C; mobile phase, stepwise gradient of acetate-sodium acetate-buffer (2.5% acetonitrile, pH 6.45) and acetonitrile:water:methanol (2:2:1), flow rate, 1.0 ml/min.

Histology: For histological analysis, the organs were immersed in 4% paraformaldehyde buffered with 0.12 M phosphate (pH 7.3) and fixed. The tissues were embedded in paraffin for histological staining with hematoxylin and eosin. The degree of spongiform neurodegeneration was scored as follows: 0, no lesions; 1, less than 20 vacuoles in the total area; 2, 20 to 100 vacuoles counted in the field at 10x magnification [field (\times 10)]; 3, clusters consisting of over 100 vacuoles spread within one field (\times 10); 4, clusters consisting of over 100 vacuoles scattered over 2 fields (\times 10); and 5, clusters of vacuoles occupying over 30% of the total area.

RESULTS

Changes in the total amount of glutamate and glutamine in the rat brain after infection of neuropathogenic viruses:

The total amount of glutamate and glutamine in the brains of the rats infected with A8, PVC211, and 57 was measured using HPLC. At 5 wpi, the total amounts of glutamate and glutamine in the cerebral hemisphere and region containing the brain stem and basal ganglia of the rats infected with neuropathogenic A8 and PVC211 viruses were no different than those in the brains of rats infected with non-neuropathogenic 57 virus (Fig. 2A, B). At 8–9 wpi, the total amounts of glutamate and glutamine in the region of the brain stem and basal ganglia of the rats infected with PVC211 were decreased compared with those in the 57-infected rats, and the total amounts of glutamine in the brain stems of the A8-infected rats were also decreased (Fig. 2D). In the cerebral hemisphere at 8–9 wpi, the total amounts of glutamine for PVC211-infected rats and total amounts of glutamate and glutamine for A8-infected rats were decreased compared with those in the 57-infected rats (Fig.

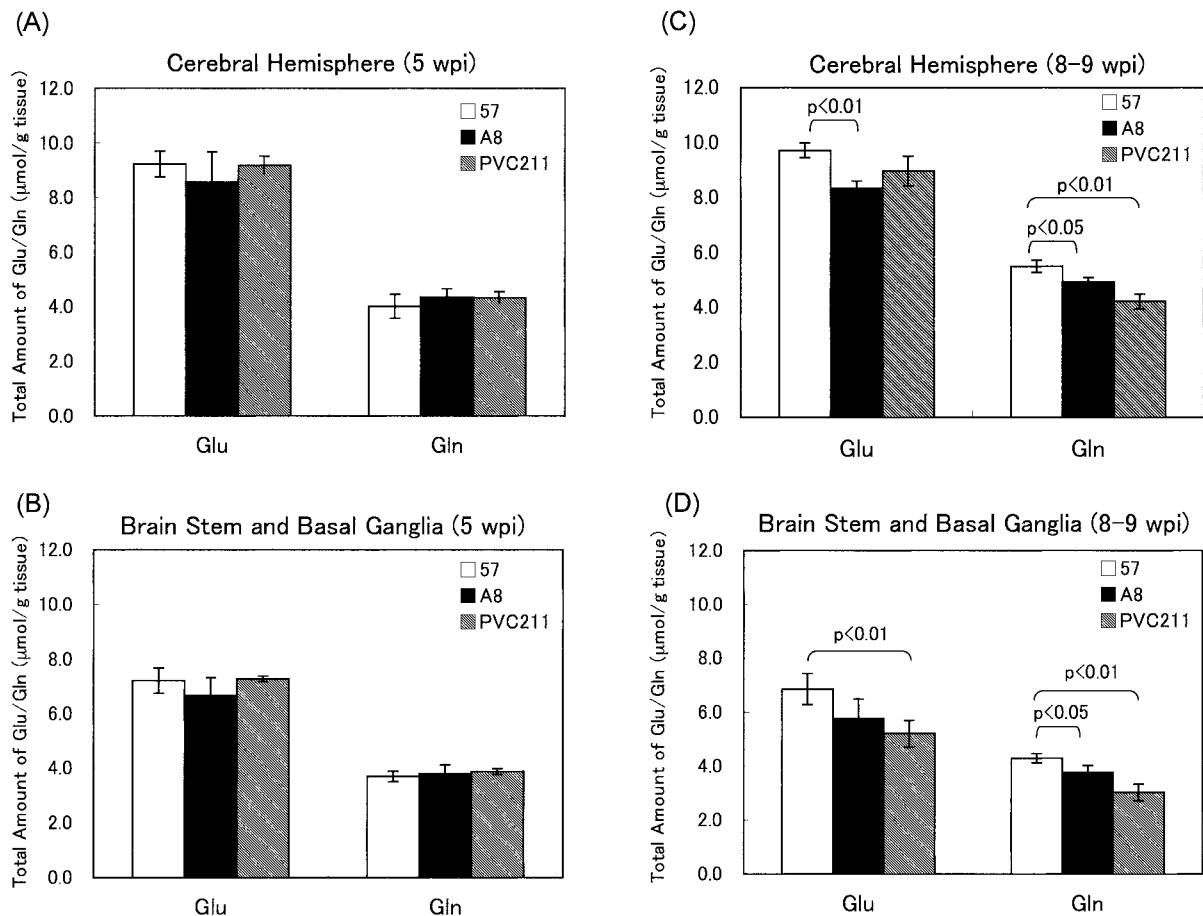


Fig. 2. The total amount of glutamate (Glu) and glutamine (Gln) in the brains of rats infected with non-neuropathogenic and neuropathogenic viruses. The amount of Glu and Gln in the cerebral hemisphere and region containing the brain stem and basal ganglia of rats infected with 57, A8, and PVC211 at 5 and 8–9 wpi was measured by high performance liquid chromatography (HPLC) using Pico-Tag methods. The values shown are the means from three rats and SEM.

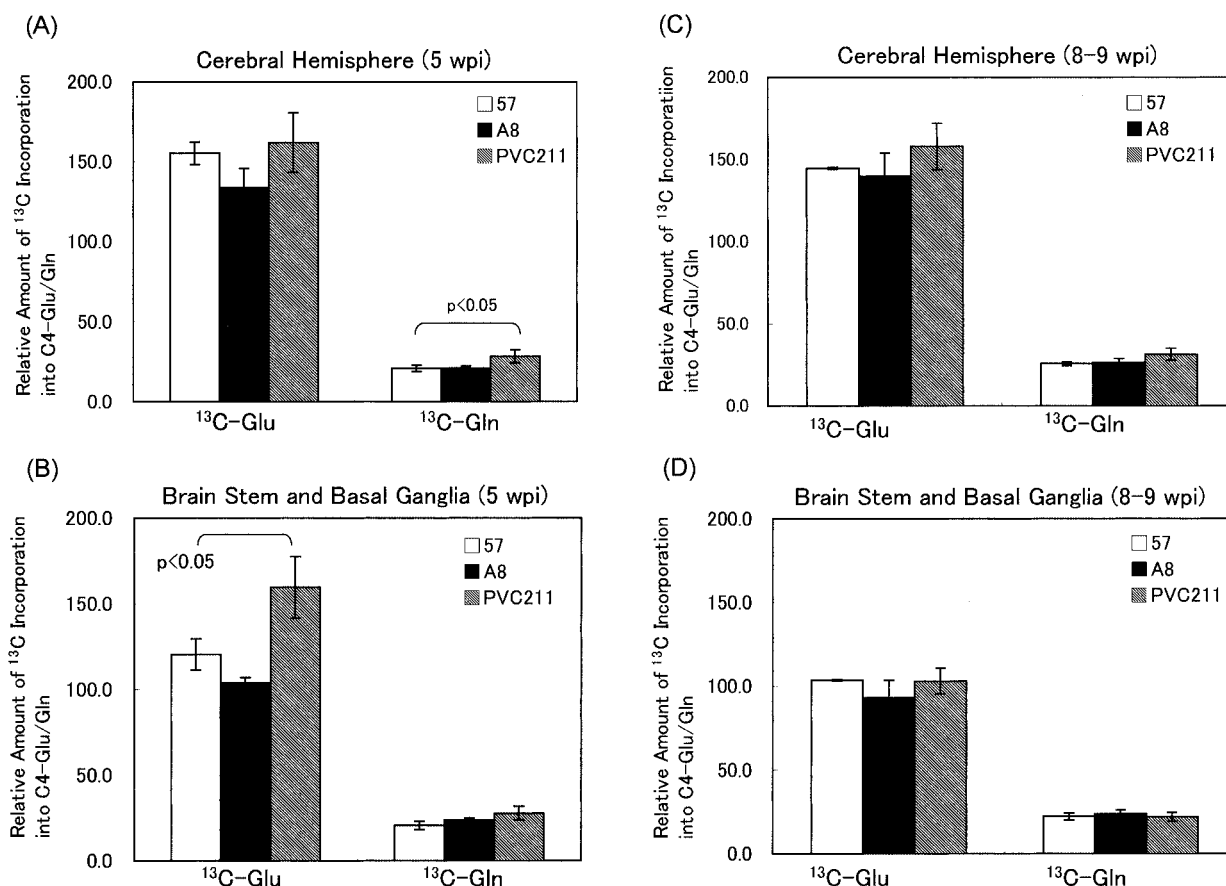


Fig. 3. The amount of ^{13}C incorporation into the C4 position of glutamate (^{13}C -Glu) and glutamine (^{13}C -Gln) in the brains of rats infected with non-neuropathogenic and neuropathogenic viruses. [$1\text{-}^{13}\text{C}$] Glucose was administered to rats infected with 57, A8, and PVC211 at 5 and 8–9 wpi, and after 15 min, the amount of ^{13}C -Glu and ^{13}C -Gln in the cerebral hemisphere and region containing the brain stem and basal ganglia was measured by ^{13}C nuclear magnetic resonance (NMR). The relative amount of ^{13}C to an external standard, sodium-3-(trimethylsilyl)-propionate-2,2,3,3- d_4 (TSP), is represented. The values shown are the means from three rats and SEM.

2C).

^{13}C -label incorporation into C4-glutamate and C4-glutamine from [$1\text{-}^{13}\text{C}$] glucose in the brains of rats infected with neuropathogenic viruses: Fifteen min after administration of [$1\text{-}^{13}\text{C}$] glucose, the amount of ^{13}C incorporation into the C4 position of the glutamate and glutamine in the brains of rats infected with A8, PVC211 and 57 was measured by ^{13}C NMR. ^{13}C -labeling to carbon atoms in cerebral metabolites after administration of [$1\text{-}^{13}\text{C}$] glucose is shown in Fig. 1. At 5 wpi, the amount of ^{13}C incorporation into C4-glutamine in cerebral hemispheres infected with PVC211 and into C4-glutamate in the region containing the brain stem and basal ganglia infected with PVC211 increased compared with those in 57-infected rats (Fig. 3A, B). At 8–9 wpi, the amount of ^{13}C incorporation into C4-glutamate and C4-glutamine in the cerebral hemisphere and region containing the brain stem and basal ganglia was comparable among the 57-, A8- and PVC211-infected rats (Fig. 3C, D).

Ratios were calculated of the amount of ^{13}C incorporation

into C4-glutamate and C4-glutamine to the total amount of glutamate and glutamine, designated as the percent ^{13}C enrichment of C4-glutamate and C4-glutamine. At 5 wpi, the percent ^{13}C enrichment of C4-glutamine in the cerebral hemisphere of the rats infected with PVC211 and that of C4-glutamate in the region containing the brain stem and basal ganglia of the rats infected with PVC211 increased compared with those in 57-infected rats (Table 1). At 8–9 wpi, the percent ^{13}C enrichment of C4-glutamate and C4-glutamine in the cerebral hemisphere and region containing the brain stem and basal ganglia of the rats infected with PVC211 increased compared with those in 57-infected rats (Table 1).

Viral replication and intensity of the lesions in the brains of rats infected with neuropathogenic viruses: Virus recovery from the brains of rats infected with A8, PVC211, and 57 at 5 and 8–9 wpi was determined (Fig. 4). At 5 wpi, the viral titers in the brains of A8- and PVC211-infected rats ranged from 3×10^4 to 2×10^5 XC-PFU/g, and that in the brains of 57-infected rats ranged from 9×10^2 to 4×10^4 XC-

Table 1. Percent ^{13}C enrichments of C4-Glu/Gln in brains after administration of $[1-^{13}\text{C}]$ glucose

Weeks Post-Infection	Region of Brain	Amino Acids	Infected Viruses		
			57	A8	PVC211
5	Cerebral Hemisphere	Glu	18.59 ± 1.75	17.25 ± 0.78	19.40 ± 1.79
		Gln	5.70 ± 0.11	5.27 ± 0.12	$7.19 \pm 0.77^{\text{a}}$
	Brain Stem and Basal Ganglia	Glu	18.39 ± 1.22	17.20 ± 1.24	$24.17 \pm 3.05^{\text{b}}$
		Gln	6.14 ± 0.84	6.90 ± 0.72	7.86 ± 1.23
8–9	Cerebral Hemisphere	Glu	16.39 ± 0.39	18.46 ± 1.69	$19.37 \pm 0.73^{\text{c}}$
		Gln	5.14 ± 0.10	5.94 ± 0.60	$8.14 \pm 0.46^{\text{d}}$
	Brain Stem and Basal Ganglia	Glu	16.70 ± 1.43	17.88 ± 2.25	$21.78 \pm 1.06^{\text{e}}$
		Gln	5.72 ± 0.74	6.98 ± 0.77	$8.03 \pm 0.47^{\text{f}}$

The percent ^{13}C enrichment of C4-Glu/Gln represents the ratio of the amount of ^{13}C incorporated into the C4 position of Glu and the C4 position of Gln to the total amount of Glu and Gln, respectively.

a) $p < 0.05$ vs Gln in cerebral hemispheres infected with 57 at 5 wpi.

b) $p < 0.05$ vs Glu in brain stems and basal ganglia infected with 57 at 5 wpi.

c) $p < 0.001$ vs Glu in cerebral hemispheres infected with 57 at 8–9 wpi.

d) $p < 0.01$ vs Gln in cerebral hemispheres infected with 57 at 8–9 wpi.

e) $p < 0.01$ vs Glu in brain stems and basal ganglia infected with 57 at 8–9 wpi.

f) $p < 0.02$ vs Gln in brain stems and basal ganglia infected with 57 at 8–9 wpi.

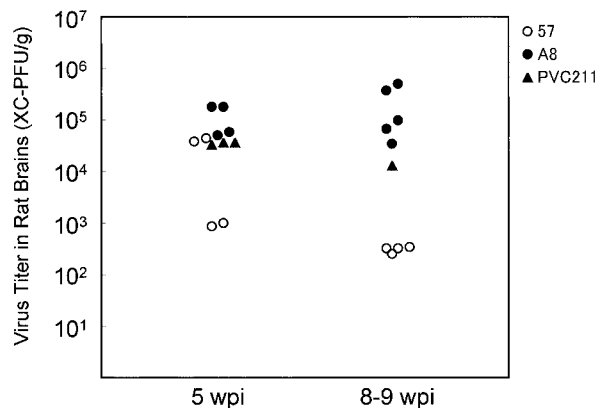


Fig. 4. Kinetics of virus growth in the brains of rats infected with A8, PVC211, and 57 viruses. Virus titers in the brains were determined by XC plaque assay.

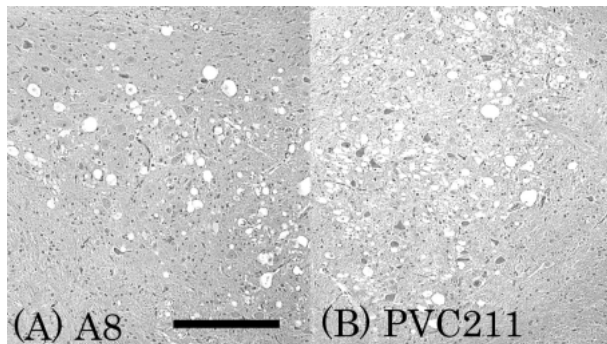


Fig. 5. Histological staining with hematoxylin and eosin 8 weeks after infection with A8 (A) or PVC211 (B). Both pictures were taken from areas around the lateral cerebellar nucleus, with lesions scored as 3. The bar indicates 250 μm .

Table 2. Intensity of the lesions in the brains of rats infected with neuropathogenic viruses

Virus	Weeks Post-Infection (Number of rats)	Spongiform Lesion in the	
		Cortex	Thalamus and Pons
A8	5 (5)	2.0 ± 0.0	2.7 ± 1.0
	8–9 (5)	2.0 ± 0.0	2.6 ± 0.4
PVC211	5 (8)	1.8 ± 0.5	3.1 ± 0.8
	8–9 (3)	1.7 ± 0.6	3.7 ± 0.6
57	5 (3)	0.0	0.0
	8–9 (18)	0.0	0.0

a) Difference is not significant ($p > 0.5$).

b) Difference is not significant ($p > 0.1$).

PFU/g. At 8–9 wpi, the viral titers in the brains of A8- and PVC211-infected rats ranged from 1×10^4 to 5×10^5 XC-PFU/g, and that in the brains of 57-infected rats were 3×10^2 XC-PFU/g.

Pathological changes with a score of 3 in the CNS of rats infected with A8 and PVC211 are shown in Fig. 5. For statistical comparison, the intensities of the lesions in the brains of rats infected with A8 and PVC211 at 5 and 8–9 wpi were also determined. There were no significant differences in the intensities of lesions in the cortex, thalamus, and pons of the rats infected with A8 and PVC211 at 5 wpi compared with those at 8–9 wpi (Table 2).

DISCUSSION

In the brains of rats infected with A8 and PVC211 at 8–9 wpi, the total amounts of glutamate and/or glutamine were decreased compared with those in 57-infected rats (Fig. 2C, D). In the brains of rats infected with 57, there was no abso-

lutely neurodegeneration despite viral replication (Fig. 4). As a possible reason for this phenomenon, it is suspected that the pool of glutamate and glutamine or *de novo* synthesis of these amino acids decreased in the brains of the rats infected with neuropathogenic viruses. It is also assumed that degradation of glutamate and glutamine was promoted in the brains of the infected-rats. To investigate the *de novo* synthesis of glutamate and glutamine in the brains of infected rats, [1-¹³C] glucose was administered into the rats infected with A8, PVC211, and 57, and after 15 min, the amount of ¹³C incorporation into the C4 position of glutamate and glutamine was measured. The amount of ¹³C incorporation into C4-glutamate and C4-glutamine in the brains of the rats infected with neuropathogenic A8 and PVC211 at 8–9 wpi was comparable to that of rats infected with non-neuropathogenic 57 (Fig. 3C, D). This result indicates that *de novo* synthesis of glutamate and glutamine does not decrease in the brains of rats infected with neuropathogenic viruses compared with the rats infected with a non-neuropathogenic virus. Therefore, most likely the pool of glutamate and glutamine in the brains of the rats infected with neuropathogenic viruses decreased and consequently, the total amount of these amino acids decreased. Degeneration or dysfunction of neuron and glial cells, which store glutamate and glutamine, by infection of neuropathogenic viruses may induce the reduction of the pool of these amino acids. It is also possible that the uptake of glutamate and glutamine into the cells is reduced by neuropathogenic virus infection.

In the brains of the rats infected with PVC211 at 8–9 wpi, the percent ¹³C enrichment of C4-glutamate and C4-glutamine, which indicates the ratio of the amount of ¹³C incorporation into C4-glutamate and C4-glutamine to the total amount of glutamate and glutamine, increased compared to the 57-infected rats (Table 1). The amount of ¹³C incorporation into C4-glutamate and C4-glutamine was unchanged after neuropathogenic virus infection (Fig. 3C, D), but the amount of total glutamate and/or glutamine decreased (Fig. 2C, D). Consequently, the percent ¹³C enrichment of C4-glutamate and C4-glutamine may have increased. Another possibility is that *de novo* synthesis of glutamate and glutamine compensatorily increases in the surviving cells after neuropathogenic virus infection. It has been revealed that glutamate and glutamine are synthesized in neurons and glial cells, respectively, and these synthesized amino acids are transferred between both cells in the synaptic region, designated the glutamate/glutamine (neuronal/astroglial) cycle [12, 14]. In the brains of rats infected with neuropathogenic viruses, this cycle is possibly abnormal because neurodegeneration is induced in the synaptic region [25], possibly resulting in accumulation of ¹³C-labeled glutamate and glutamine. To verify these possibilities, kinetic studies of metabolism and analysis in the local environment of brains are necessary.

A discrepancy was observed between the intensity of lesions and the change in cerebral metabolism. At 5 weeks after neuropathogenic virus infection, the total amount of

glutamate and glutamine in the cerebral hemisphere and region containing the brain stem and basal ganglia was unchanged, but at 8–9 weeks after virus infection, it increased (Fig. 2). Furthermore, only 5 weeks after PVC211 infection, the amount of ¹³C incorporation into C4-glutamate in the cerebral hemisphere and into C4-glutamine in the region containing the brain stem and basal ganglia increased (Fig. 3). Whereas, the intensities of the lesions in the cortex, thalamus and pons of the rats infected with A8 and PVC211 at 5 wpi were not significantly different from those at 8–9 wpi (Table 2). These results suggest that differences in cerebral metabolism can be detected, even though the degree of lesions in the brains is comparable.

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