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*Short Communication*

# The rabies virus glycoprotein determines the distribution of different rabies virus strains in the brain

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**The contribution of rabies virus (RV) glycoprotein (G) in viral distribution in the brain was examined by immunohistochemistry following stereotaxic inoculation into the rat hippocampus. Viruses used in this study include the highly neuroinvasive challenge virus standard strains (CVS-N2C and CVS-B2C) and the nonneuroinvasive attenuated SN-10 strain, as well as SN-10-derived recombinant viruses expressing the G gene from CVS-N2C (RN2C) or CVS-B2C (RB2C). The distribution of recombinant viruses in the brain was similar to those of the parental viruses from which the G was derived. For example, while CVS-B2C- and RB2C-infected neurons were seen preferentially in the hippocampus, cortex, and hypothalamus, CVS-N2C- and RN2C-infected neurons were preferentially found in the hippocampus, cortex, and thalamus. SN-10 infected efficiently almost all the brain regions. To further study the role of the RV G in virus spreading, we examined the distribution of RV antigen in brains infected with a recombinant RV in which the SN-10 G was replaced with vesicular stomatitis virus (VSV) G (SN-10-VG) was examined. The spreading of SN-10-VG to the cortex and the thalamus was drastically reduced, but the number of infected neurons in hippocampus and hypothalamus, particularly the paraventricular nucleus, was similar to the SN-10 virus. This pattern of spreading resembles that of VSV. Together, our data demonstrate that it is the G protein that determines the distribution pattern of RV in the brain.** *Journal of NeuroVirology* (2002) **8,** 345–352.

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## Introduction

Rabies virus (RV) has five structural proteins: the nucleoprotein (N), the nominal phosphoprotein (P, also termed NS), the matrix protein (M), the glycoprotein (G), and the RNA-dependent RNA polymerase (L) (Wagner and Rose, 1996). The glycoprotein (G) is the only surface protein for RV (Cox *et al*, 1977). It not only stimulates the production of virus neutralizing antibodies (Cox *et al*, 1977), but also plays a crucial role in the process of rabies pathogenesis (Dietzschold *et al*, 1996). It is believed that RV G, by binding to specific neural receptor(s) (Lentz *et al*, 1982; Thoulouze *et al*, 1998; Tuffereau *et al*, 1998), gains entry to the nervous system from the site of infection (usually by animal bites), thus contributing to the almost exclusive neurotropism (Dietzschold *et al*, 1996). The rabies virus G also mediates fusion of the viral envelope with endosomal membranes to release the infectious core into the cytosol for viral transcription and replication (Morimoto *et al*, 1992).

Once RV enters the peripheral nervous system, it travels by retrograde axonal transport (Gillet *et al*, 1986), along microtubules possibly by interacting with dynein (Jacob *et al*, 2000; Raux *et al*, 2000),

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to the nearest motor or sensory neurons in the dorsal root ganglion or anterior horn of the spinal cord, where it replicates (Iwasaki, 1991). After replication, the virus then is transported along the corticospinal tract to the brain where it infects neurons in almost all brain regions. In the nervous system, RV spreads from one neuron to another via the synaptic junctions as has been demonstrated by Ugolini (1995), Astic *et al* (1993), and Kucera *et al* (1985) using rabies virus as a transneuronal tracer. Previously, it was reported that only the infectious core, the viral ribonucleoprotein (RNP), but not the whole viral particles, are transported through synapses, because RNP-immunostaining, not viral particles, was observed in the postsynaptic boutons (Gosztonyi *et al*, 1993). This raises the question of what role is played by the surface G protein in viral spreading between neurons. The development of reverse genetics (Schnell *et al*, 1994) made it possible to engineer G-deficient RV (Mebatsion *et al*, 1996). Stereotaxic inoculation of the spike-less RV into the striatum revealed that infection of the G-deficient RV was restricted to the initially infected neurons and these virus particles could not spread to secondary neurons (Etessami *et al*, 2000). These studies demonstrate the importance of the G in the spreading of RV within the CNS.

Using the reverse genetics approach, Morimoto *et al* (2000) recently constructed mutant viruses replacing the G from neuroinvasive strains on the less pathogenic SN-10 virus. These authors found that the neurotropism of a particular RV strain was a function of its G. Furthermore, RV SN-10 strain has been used to express foreign proteins such as vesicular stomatitis virus (VSV) G protein (Foley *et al*, 2000). In the present study, we studied the distribution of these recombinant RV in the brain by stereotaxic inoculation into the rat hippocampus. It is found that the G protein determines the distribution pattern of RV in the brain.

### *Development of rabies in rats after stereotaxic inoculation of different rabies viruses into the hippocampus*

To study the distribution pattern of RV in the brain, we infected Sprague–Dawley rats (5 to 6 weeks of age) stereotaxically with $10^5$ focus forming unit (ffu) of one of the following viruses: (1) the parental viruses CVS-N2C, CVS-B2C that were derived from CVS-24 by passaging in different cell lines (Morimoto *et al*, 1998); (2) the parental virus SN-10 virus that was derived from SAD B19 as described previously (Schnell *et al*, 1994); (3) the SN-10-333 mutant virus that has an $arg^{333}$ to $glu^{333}$ mutation (Morimoto *et al*, 2001); (4) the recombinant RV RB2C, which is a SN-10 virus in which the G was replaced with the G of CVS-B2C (Morimoto *et al*, 2000); (5) RB2C-333 containing the CVS-B2C G gene with an $arg^{333}$ to $glu^{333}$ mutation (Morimoto *et al*, 2001); (6) the recombinant RV RN2C expressing the G from CVS-N2C (Morimoto *et al*, 2000); (7) the recombinant RV RN2C-333 expressing the G from CVS-N2C with an $arg^{333}$ to $glu^{333}$ mutation (Morimoto *et al*, 2001); and (8) the recombinant RV SN-10-VG expressing a chemeric G gene that consists of the ecto- and transmembrane domains of VSV G fused to the cytoplasmic tail of SN-10 G (Foley *et al*, 2000). The construction, cultivation, and titration of these viruses have been described previously (Schnell *et al*, 1994).

**Table 1** Development of rabies in rats after stereotaxic inoculation into the hippocampus

| *Virus* | *Numbers of animals that showed clinical signs of rabies at day 7 p.i. (morbidity in %)* |
|---|---|
| CVS-B2C | 3/3 (100) |
| RB2C | 3/3 (100) |
| RB2C-333 | 1/3 (0) |
| CVS-N2C | 3/3 (100) |
| RN2C | 3/3 (100) |
| RN2C-333 | 0/3 (0) |
| SN-10 | 0/3 (0) |
| SN-10-333 | 0/3 (0) |
| SN-10-VG | 1/3 (33) |

Stereotaxic surgery and virus inoculation into the hippocampus was performed using the following coordinates: 3.2 mm ventral, 1.6 mm lateral, and 2.8 mm posterior (Paxinos and Watson, 1986) after the rats were anesthetized with sodium pentobarbital. Three rats were infected with each of the virus strains. The animals were observed daily for development of clinical diseases during a 7-day period. Table 1 summarizes the clinical signs at day 7 postinfection (p.i.). Overall, the clinical signs of rats infected with recombinant viruses expressing CVS G proteins were similar to those infected with the parental virus from which the G was derived except those viruses with mutation of arginine 333. Only one rat infected with recombinant virus RB2C-333 with mutation at arginine 333 of the G developed clinical signs at day 7. One rat infected with SN-10-VG also developed clinical signs at day 7, whereas none of the animals infected with SN-10 or SN-10-333 showed clinical signs of rabies at day 7 p.i.

### *Distribution of RV antigen in the brain*

To study the distribution of RV in the brain, rats were anaesthetized and perfused transcardially with 10% neutrally buffered formalin at day 7 after infection. After perfusion, the brains were removed for immunohistochemistry. Coronal sections (50 $\mu$m thickness) were prepared from the perfused rat brain with a Vibratome (TPI, St. Louis, MO). Rabies virus antigen was detected by immunohistochemistry using anti-N monoclonal antibody (Mab 802-2) (Yan *et al*, 2001). Expression of viral antigen was examined in the hippocampus (HIP), cortex (CTX), amygdala (AMY), thalamus, and hypothalamus. In the thalamus, the following nuclei were examined for the presence

**Table 2** N antigen in brains infected with rabies virus

| *Virus* | *CTX* | *AMY* | *HIP* | *MHb* | *LD* | *VPM* | *VPL* | *VL* | *Rt* | *DHN* | *VMN* | *PVN* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CVS-B2C | ++ | ++ | ++ | ++ | + | + | + | + | + | ++ | ++ | ++ |
| RB2C | +++ | +++ | +++ | ++ | + | + | + | + | + | +++ | +++ | +++ |
| RB2C-333 | +++ | +++ | +++ | ++ | + | + | + | + | + | ++ | ++ | +++ |
| CVS-N2C | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + |
| RN2C | +++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + |
| RN2C-333 | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + |
| SN-10 | +++ | + | ++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | +++ |
| SN-10-333 | — | — | + | — | — | — | — | — | — | — | — | — |
| SN-10 VG | + | + | ++ | + | + | + | + | + | + | + | + | +++ |

+ 1–5 positive neurons per view area.
++ 6–20 positive neurons per view area.
+++ more than 20 positive neurons per view area (at 200× magnification).

of viral antigen: medial habenular nucleus (MHb), leterodorsal thalamus nuclei (LD), ventrolateral thalamus nucleus (VL), ventral posteromedial thalamus nucleus (VPM), ventral posterolateral thalamus nucleus (VPL), and reticular thalamus nucleus (Rt). In the hypothalamus, the following nuclei were examined for virus antigens: posterior hypothalamus nucleus (PHN), dorsomedial hypothalamus nucleus (DHN), ventromedial hypothalamus nucleus (VMN), and paraventricular nucleus (PVN). As summarized in Table 2, viral antigen was detected in almost all the brain areas monitored for all the viruses except SN-10-333.

Rabies virus antigen was only detected in neurons. The intensity of the immunostaining in the brain of rats infected with the recombinant viruses was similar to that seen in brains infected with the parental viruses from which the G was derived. For example, strong immunostaining of viral antigens was observed in almost all neurons in the hippocampus in rats infected with CVS viruses (CVS-N2C and CVS-B2C) and recombinant viruses with G derived from CVS viruses (RN2C, RN2C-333, RB2C, and RB2C-333) (Figure 1A). The immunostaining was less intense in the hippocampus in rats infected with SN-10 virus. All the CVS viruses and SN-10 showed strong immunostaining in the cortex (Figure 1B). In the thalamus, viral antigens were detected in many of the nuclei, particularly MHb, LD, including LD dorsomedial (LDDM) and ventrolateral nuclei (LDVL), VL, VPM, VPL, and Rt in animals infected with SN-10 and CVS-N2C as well as recombinant viruses expressing CVS-N2C G protein (Figure 1C). Viral antigen was preferentially detected in the MHb, and to a much lesser extent in other nuclei, in rats infected with CVS-B2C and RB2C. Figure 1D shows the immunostaining of rabies virus N antigen in the hypothalamus, particularly in the PVN. Rats infected with CVS-B2C, RB2C, or RB2C-333 showed more viral antigen in the PVN than animals infected with CVS-N2C, RN2C, or RN2C-333. Strong immunostaining of viral antigen was observed in the PVN of rats infected with SN-10. In SN-10-VG virus-infected brain, the overall staining patterns were similar to SN-10 virus-infected brain in the hippocampus and PVN, although much less viral antigen was detected in the cortex and thalamus of SN-10-VG virus- than SN-10 virus-infected brain (Figures 1A–D). These distribution patterns were similar in all the three rats infected with the same virus (data not shown). All the data suggest that distribution of rabies virus within the brain at day 7 after infection is largely dependent on the origin of the G protein.

### *Effects of the mutation from arg$^{333}$ to glu$^{333}$ of the G on RV distribution in the brain*

Arg$^{333}$ of the G has been reported as a hallmark of virulence for RV (Dietzschold *et al*, 1983; Seif *et al*, 1985). To study the effect of virus attenuation on virus spread within the CNS, the arg$^{333}$ of the G of SN-10, RN2C, and RB2C was mutated to glu$^{333}$ (Morimoto *et al*, 2000, 2001). This mutation had a dramatic effect on the spreading of the SN-10 virus and resulted in almost complete inhibition of viral antigen expression (see SN-10-333 in Figure 1). Viral antigen was observed only in a few neurons in the hippocampus at the site of inoculation when examined under higher magnification (data not shown). In contrast, the same arg$^{333}$ to glu$^{333}$ mutation in the G of RB2C or RN2C did not change the distribution pattern (see RN2C-333 and RB2C-333 in Figure 1), indicating that only in the case of SN-10 G arg$^{333}$ determines the ability of the virus to spread within the CNS.

### *Viral antigen distribution in the brain for recombinant virus expressing VSV G*

To further determine the role of G in RV spreading within the CNS, we infected rats with SN-10-VG in which the SN-10 G was replaced by VSV G and examined the distribution of viral antigens in the brain under higher magnification. Although the distribution of viral antigens in the hippocampus and hypothalamus, particularly PVN, was similar for both viruses, the number of positive neurons in the cortex and thalamus was much lower in rats infected with SN-10-VG than with SN-10 (Figure 2). These observations further confirm that the distribution pattern of RV in the brain is determined by epitopes of the G protein.

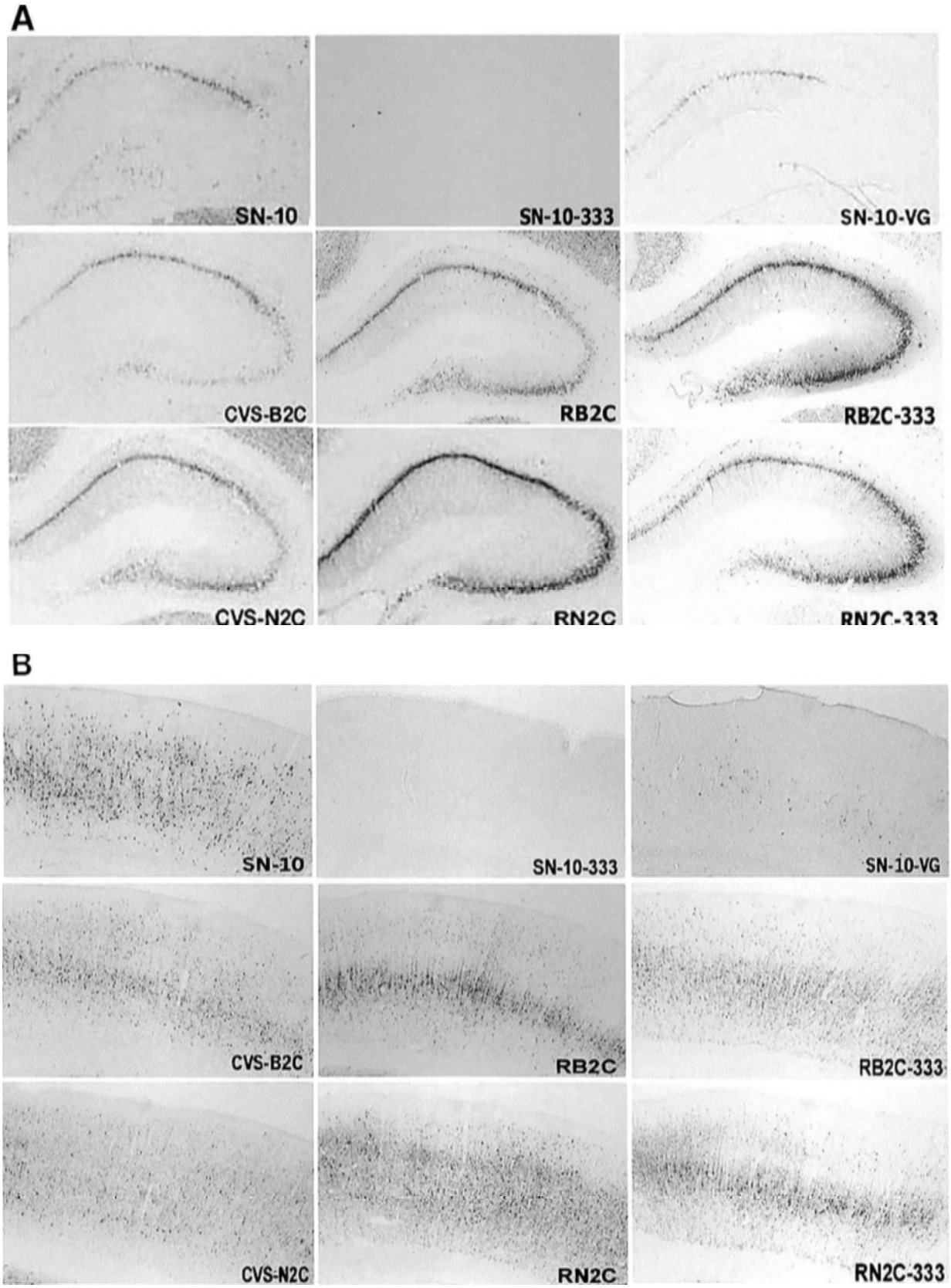


**Figure 1** Viral antigen distribution in different areas of the brain. Rats were infected with different rabies viruses. Rats were perfused and the brains were taken for immunocytochemistry with anti-rabies virus N antibody 802-2. Viral antigens were examined in the hippocampus (**A**), cortex (**B**), thalamus (**C**), and hypothalamus (**D**).

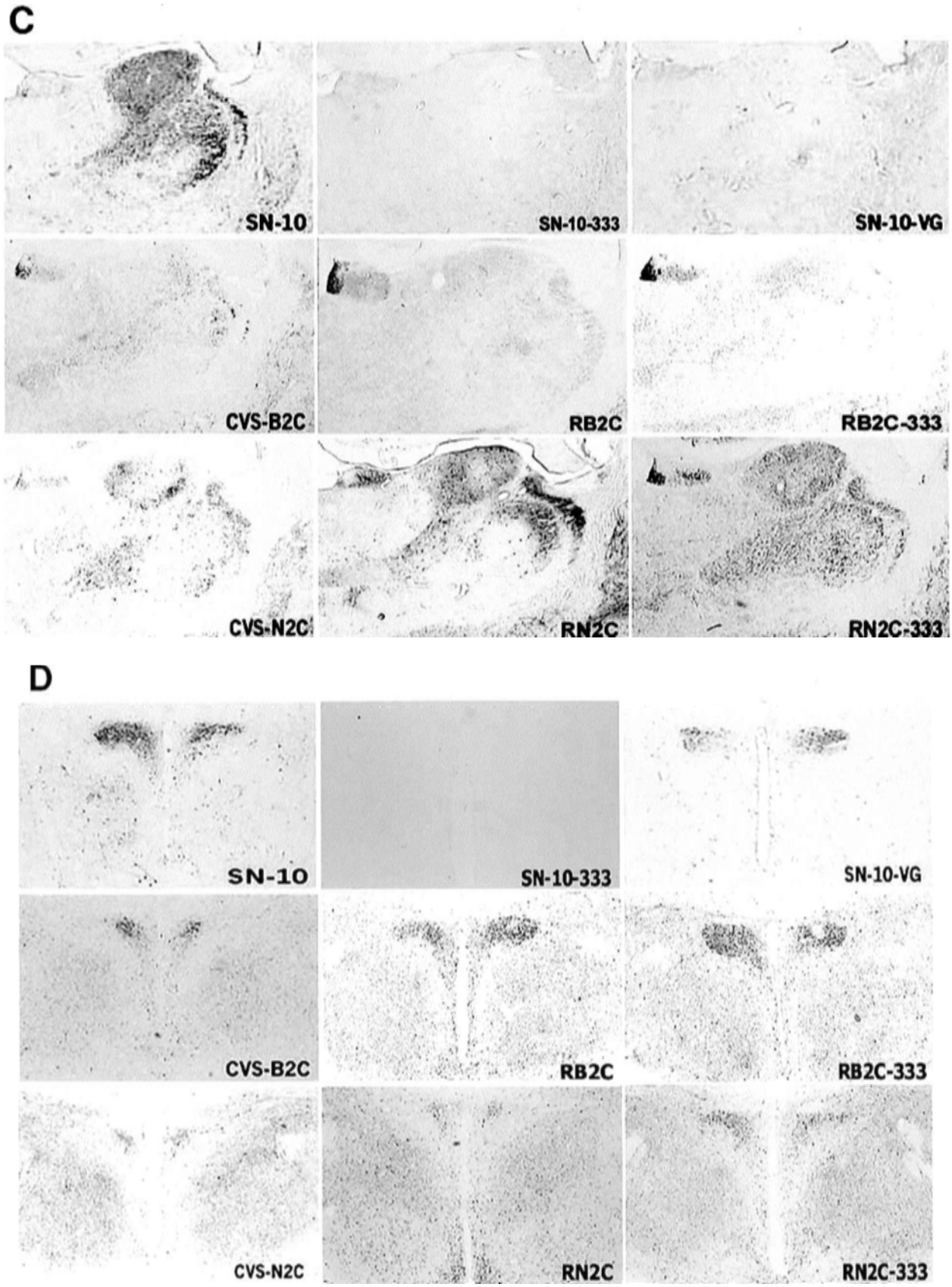


**Figure 1** (Continued).

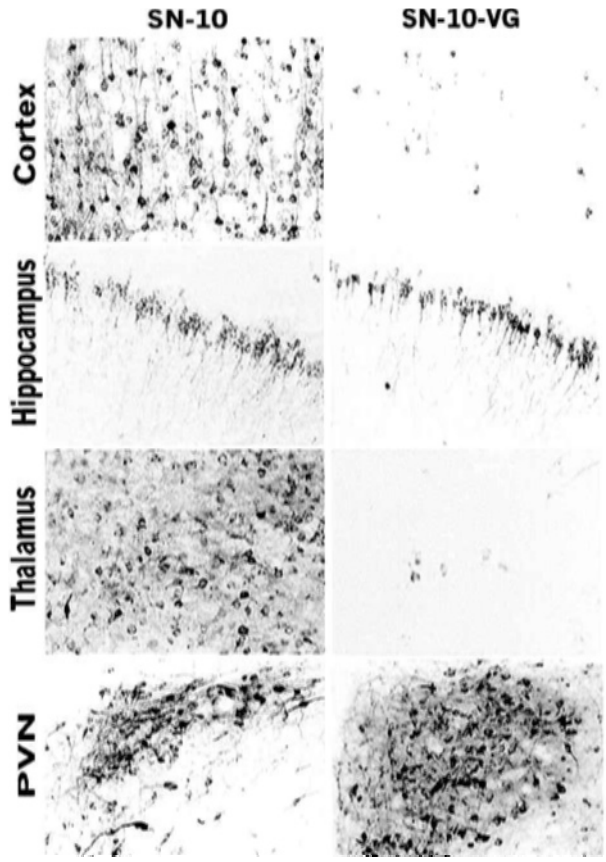


**Figure 2** Comparison of viral antigen distribution in the brain by SN-10 and SN-10-VG. Rats were infected with either SN-10 or SN-10-VG, perfused, and subjected to immunocytochemistry as for Figure 1. Shown here are viral antigens in the cortex (cerebral cortex), hippocampus (CA1), thalamus (VPM), and PVN.

RV G has been reported to be largely responsible for rabies pathogenesis (Dietzschold *et al*, 1996). RV G, by interacting with neuronal receptor(s), contributes to the high neurotropism of the virus (Lentz *et al*, 1982; Thoulouze *et al*, 1998; Tuffereau *et al*, 1998). RV G is also a major determinant of RV pathogenicity (Morimoto *et al*, 2000). It is well known that mutation on the G, particularly at $arg^{333}$, can dramatically reduce the pathogenicity of a particular RV strain (Dietzschold *et al*, 1983; Seif *et al*, 1985). In addition, RV with deleted G gene cannot invade neurons (Etessami *et al*, 2000). Furthermore, the level of G expression has been correlated with the induction of apoptosis both *in vitro* (Morimoto *et al*, 1999) and *in vivo* (Yan *et al*, 2001). In the present study, we found that rabies virus G also determines the distribution pattern of a particular virus strain in the brain.

Viral antigen detection in the brain revealed that the distribution pattern of the recombinant viruses is similar to the parental virus from which the G protein was derived. For example, neurons infected with recombinant viruses expressing CVS-B2C G (RB2-C and RB2C-333) were found preferentially in the hippocampus, cortex, and PVN, just like the parental virus CVS-B2C; while these viruses infected only a few neurons of the thalamus. On the other hand, recombinant viruses expressing CVS-N2C G (CVS-N2C, RN2C, and RN2C-333) infected strongly the neurons of the thalamus. In contrast, these viruses spread less efficiently to the PVN. Despite the fact that all the recombinant viruses contained four of the five viral proteins derived from SN-10, their distributions differ markedly from that of SN-10. SN-10 virus (also SN-10-VG) infected efficiently all brain regions including thalamus and hypothalamus. However, the expression of viral antigen was much less intensive in the hippocampus, particularly in the CA3 and CA4 regions, as compared to the CVS viruses. Our data suggest that rabies virus G determines the distribution pattern in the brain. The observed disparities in virus distribution could be due to differences in the susceptibility of particular neuron populations for the various RVs.

To further examine the role of RV G in viral spreading within the CNS, a recombinant RV (SN-10-VG) in which the RV G was replaced by VSV G was used. Virus distribution of SN-10-VG in the hippocampus was similar to that observed with SN-10. However, the spreading of SN-10-VG to cortex and thalamus was much less pronounced than that observed with SN-10. On the other hand, viral antigen staining in PVN of rats infected with SN-10-VG is similar to that in rats infected with SN-10. These distribution pattern of SN-10-VG is similar to that reported for VSV. In the VSV-infected brain, cortical and thalamus neurons are much less involved in virus dissemination (Miyoshi *et al*, 1971; Huneycutt *et al*, 1994; Plakhov *et al*, 1995). Similar immunostaining was detected in the hippocampus in mice infected with SN-10-VG as SN-10 because both viruses were inoculated directly into the hippocampus. The data further suggest that the distribution pattern of RV in the brain is largely dependent on the G protein.

$Arg^{333}$ of the G has been reported to play an important role in rabies pathogenesis (Dietzschold *et al*, 1983; Seif *et al*, 1985). RV strains (ERA or CVS-11) with mutation of $arg^{333}$ are nonpathogenic in adult mice even after intracerebral inoculation (Dietzschold *et al*, 1983), are unable to infect motoneurons *in vivo* and *in vitro* (Coulon *et al*, 1998), and do not spread efficiently in neuroblastoma cells *in vitro* (Dietzschold *et al*, 1987), and in neurons *in vivo* via inoculation into the anterior chamber of the eye (Kucera *et al*, 1985). However, when stereotaxically inoculated into the striatum and cerebellum of adult mice, Av01 virus with a G mutation on $arg^{333}$ from CVS-11 spread within the CNS and induced rabies (Yang and Jackson, 1992). In our study, three engineered viruses with the $arg^{333}$ changed to $glu^{333}$ (Morimoto *et al*, 2000) were inoculated stereotaxically into the hippocampus of rats. The $arg^{333}$ to $glu^{333}$ mutation in SN-10 G abolished completely the neuronal spread of the virus and viral antigen was only observed in the originally infected

neurons in the hippocampus, a pattern similar to that infected stereotaxically by the G-deficient virus into the striatum (Etessami *et al*, 2000). In contrast, mutation of $arg^{333}$ to $glu^{333}$ of the G in RB2C or RN2C did not change the pattern of viral spreading. RB2C-333 spreads to hippocampus, cortex, and PVN as efficiently as the parental virus RB2C. Likewise, RN2C-333 spreads to hippocampus, cortex, and thalamus as efficiently as the parental virus RN2C. Our data, thus, indicate that only in the case of SN-10 G, $arg^{333}$ determines the ability of the virus to spread within the CNS. Although mutation of $arg^{333}$ to $glu^{333}$ of the G in RB2C or RN2C did not change the distribution pattern, only one rat infected with RB2C-333 developed mild clinical signs at day 7 p.i. It will be interesting to see if these animals will eventually develop rabies if left for a longer period. It will also be interesting to see if the distribution of viral antigens in these animals correlates with pathological changes, particularly inflammatory responses. In our previous study (Yan *et al*, 2001), we presented evidence that the little inflammation was observed in the parenchyma of mice infected with either laboratory-adapted or wild-type rabies virus apart from a mild leptomeningitis. However, Hooper *et al* (1998) observed strong inflammatory responses in mice infected with CVS-F3, a virus with mutation of $arg^{333}$ to $glu^{333}$ on the G. It is possible that inflammatory responses are responsible for the lack of virus spreading in animals infected with virus SN-10-333.

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