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Short Communication

Differential use of the nicotinic receptor by rabies virus based upon substrate origin

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To determine the role that the neuronal nicotinic acetylcholine receptor plays in the adsorption process of rabies virus (RV), adult dorsal root ganglion dissociated cultures were exposed to nicotinic agonists before being inoculated. The fixed strain of RV Challenge Virus Standard-11 (CVS-11) was used after being passaged in two different ways, in baby hamster kidney (BHK) cells and in adult mouse brain (MB). Carbachol and nicotine reduced the percentage of CVS-MB infected neurons, yet none of the agonists tested changed the proportion of CVS-BHK infected neurons. This result suggests that the RV phenotype changes depending on its replication environment and neuronal nicotinic acetylcholine receptors are preferentially used for infection by RV strains adapted to adult mouse brain but not to fibroblasts. *Journal of NeuroVirology* (2002) **8,** 150–154.

Keywords: rabies virus; adult sensory neuron; nicotinic receptor; viral receptor; nicotinic agonists; neuron culture

Infection by RV inevitably leads to the occurrence of lethal encephalitis. Attachment of RV to the target cell represents the first step in the *in vivo* and *in vitro* infection process. After entering a peripheral nerve, RV is retrogradely transported to the central nervous system where replication takes place, causing the classical rabies symptoms (Plotkin, 2000). The presence of high affinity receptors for RV accounts for its marked neurotropism (Haywood, 1994). There is evidence that RV might use certain membrane molecules during adsorption, including carbohydrates such as sialic acid (Conti *et al*, 1986), proteins such as the nicotinic acetylcholine receptor (Gastka *et al*, 1996), neural cellular adhesion molecule (Thoulouze *et al*, 1998), the low-affinity neurotrophin receptor (p75 NTR) (Tuffereau *et al*, 1998), or another protein not yet identified in BHK cells (Broughan and Wunner, 1995).

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Several reports suggest that the nicotinic acetylcholine receptor might be involved in the adsorption process of RV to neurons. Pretreatment of muscle cells with the nicotinic acetylcholine receptor antagonist α-bungarotoxin (α-BGT) has been reported to decrease their susceptibility to infection (Tsiang *et al*, 1986). The rabies virus glycoprotein (VG) and α-BGT share some amino acid sequences (Lentz, 1991). Monoclonal antibodies directed against a synthetic tetradecapeptide with sequence similarity to the presumed virus binding site in target cells recognizes both VG and α-BGT and prevents their binding to the nicotinic receptor (Bracci *et al*, 1998). In addition, anti-idiotypic antibodies directed against anti-VG monoclonal antibodies recognize nicotinic receptors (Hanham *et al*, 1993).

In a previous report, we showed that the nicotinic antagonists D-tubocurarine and mecamilamine reduced viral infection in cultured dorsal root ganglion neurons from adult mice (Castellanos *et al*, 1997). Nevertheless, participation of the nicotinic receptor in the rabies virus infection process is controversial because cells lacking this receptor can also be infected by the virus, whereas other cells bearing it do not alter their susceptibility to infection when treated with cholinergic antagonists (Reagan and Wunner, 1985).

It is well known that various genetically and phenotypically different viruses coexist in laboratory viral suspensions. They replicate differentially depending on the cellular environment where infection occurs, giving rise to selection for the most competent viruses in each tissue (Morimoto *et al*, 1998). The goal of this work is to describe the susceptibility of cultured adult mouse dorsal root ganglion cells pretreated with nicotinic agonists to infection with two CVS rabies strains, one replicated in BHK-21 cells and the other in adult mouse brain.

Materials and methods

Dorsal root ganglion cells were dissociated and cultured according to standard procedures in our laboratory (Castellanos *et al*, 1997; Castellanos and Hurtado, 1999). In short, dorsal root ganglia were obtained from adult ICR mice (25 g), incubated at 37°C with collagenase (190 U/ml), and mechanically dissociated with progressively narrower fire-polished Pasteur pipettes. We used Dubecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Complete Medium, CM) to resuspend and plate the cells on poly-L-lysine (10 μg/ml) coated glass coverslips at a density of 40,000 cells/ml. Cultures were maintained in a humidified incubator at 37°C with a mixture of 95% air/5% CO_2. Culture medium containing 10 μM cytosine arabinoside was replaced every 48 h. At day 5, medium was replaced by CM containing the different nicotinic agonists described later and incubated for 2 h. Cultures were washed once with CM and the viral inoculum was added and incubated for 1 h. Cells were washed twice with CM and then incubated for 48 h in CM. The agonists tested were nicotine, cytisine, carbachol, acetylcholine, dimethyl-phenyl piperazinium and lobeline, at 0.1 μM, 1 μM, and 10 μM, concentrations at which an electrophysiological effect is obtained without causing detectable harm to the cells (Dolezal *et al*, 1995). CVS-11 adapted to mouse neonatal brain (kindly donated to Colombian NIH by the PAHO/WHO) was used to produce two different virus strains: BHK-21 adapted CVS (CVS-BHK) and adult ICR mouse brain-adapted CVS (CVS-MB) at $10^{1.5}$ and $10^{4.3}$ LD_{50} titers, respectively. Produced virus was titrated by calculating the LD_{50} (lethal dose 50) after intracerebral injections of increasing dilutions in young ICR mice (14–16 g). Viral dilutions were prepared from stocks to yield a multiplicity of infection (MOI) of 0.025 for the CVS-BHK strain and 0.03 for the CVS-MB strain, with the purpose of decreasing unspecific interactions.

For immunodetection of infected cells, cultures were washed with PBS and fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. Endogenous peroxidase was quenched with 0.3% H_2O_2 in PBS. Hamster antirabies serum (1:900) prepared in antibody buffer (0.05 M Tris, 0.1% Triton X-100, 0.5 M NaCl pH 8.6) was added for 30 min, washed three times with PBS, and secondary antibody was added (biotinilated anti-hamster, 2.5 μg/ml) for 30 min. Cells were further incubated with the avidin-biotinilated peroxidase complex and then reacted for 4 min with a suspension containing 1 mg/ml 3′3-diaminobenzidine-HCl and 0.02% H_2O_2. Cultures were counterstained with Mayer's hemalun and mounted with Poly-Mount. Cultures were performed in triplicate and each agonist concentration and each culture was repeated four times. Five hundred cells including neuronal and nonneuronal cells (infected and noninfected) were blindly and randomly counted at 400× magnification in each glass coverslip. Neurons were identified according to the morphological properties previously described: in short, spheroid and large cell bodies with one or many neurites (Ninomiya *et al*, 1994; Castellanos *et al*, 1996). Cells were counted and the proportion of neuronal and nonneuronal cells for the three dilutions for each drug and control were calculated. This was followed by an arcsine transformation in order to do a one-way ANOVA test and a LSD-test (Sokal and Rohlf, 1981).

Results

Several nonneuronal cells (Schwann cells and fibroblasts) and neurons of different diameters were observed in culture (Figure 1A). All three types of cells in culture were infected with the virus (Figure 1B) and no immunoreactivity was observed in noninfected control cultures. Both viruses showed neurotropism in control cultures (without drug), because a higher percentage of infected cells were neurons (Table 1). The proportion of infected neurons and nonneuronal cells was 14.4% and 8.9% for CVS-BHK and 12.3% and 7.8% for CVS-MB, respectively.

In CVS-BHK-infected cultures, the proportion of infected neurons was not significantly different between agonist-treated and control cultures ($P > 0.05$). In CVS-MB-infected cultures, carbachol (1 μM) and nicotine (0.1 μM, 1 μM) significantly reduced the proportion of infected neurons compared to control ($P < 0.05$) (Table 2). Cytisine, acetylcholine, lobeline, and dimethyl phenyl piperazinium did not change the proportion of infected neurons. Nicotine (10 μM) and carbachol (10 μM, 0.1 μM) decreased the level of infection in a nonsignificant manner. The proportion of infected nonneuronal cells with both types of virus was not affected by treatment with agonists ($P > 0.05$), an observation consistent with the absence of nicotinic receptors on these cell types and the absence of drugs toxic effects.

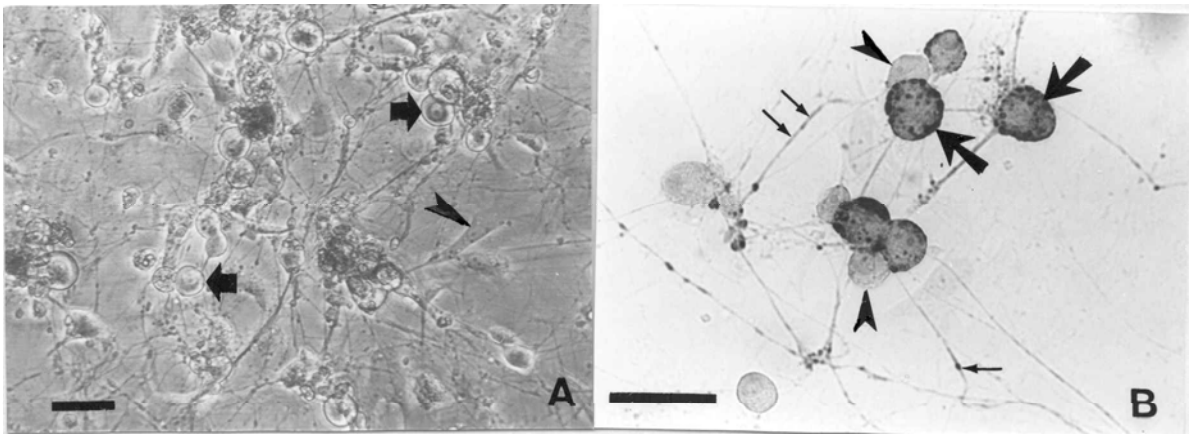

Figure 1 (**A**) Phase contrast micrography of adult dorsal root ganglion cultured cells. Note the round and refringent neuronal somas (arrow), and the nonrefringent nonneuronal cells (arrow head). (**B**) Indirect immunoperoxidase detection of RV-infected cells. Large neurons (large arrows) and some neurites (little arrows) are positive for infection. Noninfected small neurons are also observed (arrow head). Scale bar, 50 μm.

Discussion

In this work, we show that nicotinic agonists reduce the number of adult sensory neurons infected by RV when it has been adapted to adult mouse brain but not to cultured fibroblast cells. Both virus strains showed classical neurotropism in this primary culture system. Several reports have demonstrated that dorsal root ganglion cultures from embryonic (Licke and Tsiang, 1987; Tsiang *et al*, 1989) and adult mice (Castellanos *et al*, 1996) are susceptible to infection with CVS rabies virus. In addition, nicotinic receptor mRNA and protein has been detected in sensory neurons (Sucher *et al*, 1990; Boyd *et al*, 1991). It has also been shown that RV uses the nicotinic acetylcholine receptor during the infection process (Tsiang *et al*, 1986; Bracci *et al*, 1988; Lentz, 1991; Hanham *et al*, 1993; Castellanos *et al*, 1997).

Our findings support the idea that RV uses the nicotinic receptor in its adsorption process. There is a wide diversity of neuronal nicotinic receptors with different pharmacological and biochemical properties, including multiple agonist sensitivity and rank-order potencies, depending on subunit combinations (McGehee, 1999). The nicotinic receptor subunits known to be present in sensory neurons are α3, α4, α5, α7, α8, and β2 and β4, with a high predominance of α3β4 (Boyd *et al*, 1991; Nelson and Lindstrom, 1999; Roth *et al*, 2000). Thus, it is possible that in our cultures, different levels of expression of these subunits is manifested in different RV binding affinities, providing a reasonable explanation for the finding that not all of the drugs used had an effect on the infection process. We found that the lowest concentration (0.1 μM) of nicotine reduced the infection level by half whereas the highest (10 μM) did not have an effect. Similarly, carbachol produced a comparable effect at 1 μM whereas the highest concentration (10 μM) did not result in a larger inhibition, nor did it change the proportion of infected neurons. It could be that high nicotinic agonist concentrations hyperexcite the neurons, which may facilitate the infection or the viral replicative process. It was reported that nerve growth factor (NGF) and neurotrophin 3 (NT-3) reduce the proportion of infected DRG neurons in culture (Castellanos *et al*, 2000). The inhibition is higher at lower concentrations (NGF 2 and 10 ng/ml and NT-3 1 and 5 ng/ml) while higher concentrations had no effect. The lack of a dose-dependent effect in this case could be due to a change in the metabolic state of neurons expressing NGF and NT-3 receptors, which makes the infection process harder. In that report and here as well, a toxic effect was ruled out by counting the total number of neurons per field both in control and test conditions. Carbachol and nicotine might be having a metabolic effect that at high doses does not affect virus adsorption or replication. It is also possible that the lack of a

Table 1 Neuronal and nonneuronal cell infection in dorsal root ganglion cultures using two rabies virus strains[a]

	CVS-BHK		*CVS-MB*	
	Noninfected	*Infected*	*Noninfected*	*Infected*
Neurons	1132 (85.6%)	191 (14.4%)	1231 (87.7%)	172 (12.3%)
Nonneuronal cells	4274 (91.1%)	418 (8.9%)	4550 (92.2%)	384 (7.8%)

[a]Neurotropism was determined by χ^2 test between infected neuronal and nonneuronal cell proportions ($P < 0.001$). There are no significant differences between the proportion of infected neurons with both strains of virus at the MOI used ($P > 0.05$).

Table 2 Rabies virus infection inhibition of adult mouse DRG neurons

Agonist (concentration)	*Percentage of inhibition (Mean ± SEM)*	
	CVS-MB	CVS-BHK
Nicotine (0.1 μM)	55.1 ± 11.2[a]	13.2 ± 8.5
Nicotine (1 μM)	43.3 ± 8.8[a]	7.8 ± 5.6
Nicotine (10 μM)	22.8 ± 4.5	5.2 ± 7.1
Carbachol (0.1 μM)	29.1 ± 5.7	11.1 ± 9.6
Carbachol (1 μM)	47.0 ± 9.2[a]	6.2 ± 6.8
Carbachol (10 μM)	20.6 ± 4.1	7.8 ± 6.3

Percentages of inhibition by nicotinic agonists.
[a]Significant differences with agonist-untreated control ($P < 0.05$) tested by ANOVA followed by a LSD test.

dose-dependent effect could be due to the participation of other receptors such as NCAM and p75 during the adsorption process (see next).

Rabies virus has a strong neurotropism both *in vivo* and *in vitro*, but the laboratory-adapted strains have the capacity to infect a wide variety of cells regardless of origin. Both laboratory-fixed virus strains, fibroblast or brain-adapted, would behave differently when infecting cells by using different receptor molecules for the first steps of binding. Thus, it could be possible to get a differential infection block. It is known that street rabies virus can be isolated on neuroblastoma cells rather than BHK-21 monolayers (Rudd and Trimarchi, 1987), suggesting that nerves-derived cells express either the right receptors or a variety of them in order to achieve high infection levels by certain strains.

Furthermore, neuronal or fibroblast-passaged viruses have distinctive biological properties, as demonstrated by Morimoto and coworkers (1998), who found that CVS exists as quasispecies complexes, each with characteristic genome and proteins. There is a virus variant that shows preference for neuronal cells and can be isolated by favoring its replication in an appropriate environment. The phenotype of the harvested virus also changes when the inoculum is made on adult or suckling mouse brains. Additionally, it was shown that several host proteins are incorporated in virus derived from cell lines (Sagara *et al*, 1997). It could be possible that specific sugars and lipids of each cellular substrate are also incorporated, modifying the virus-binding properties. These putative differences in structure and composition of rabies virus strains might explain the reduction in the level of neuronal CVS-MB infection but not of neuronal CVS-BHK infection by nicotinic agonists. It could also explain why Lentz *et al* (1997) observed that carbachol and nicotine did not reduce the level of RV infection of IMR-32 cells *in vitro* and α-BGT did, results different from our findings. The virus used in that study was a CVS strain passaged in mouse neuroblastoma (N18) cells and the exposure to the virus was longer than in ours. We find it interesting that $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$ and $\beta 2$, $\beta 4$ subunits cDNAs were isolated from IMR-32 cells (Groot-Kormelink and Luyten, 1997), but electrophysiological studies revealed primarily $\alpha 3\beta 4$-type currents (Nelson and Lindstrom, 1999). $\alpha 3\beta 4$ receptors are insensitive to α-BGT, but α-BGT reduced RV infection in these cells, yet mecamylamine and D-tubocurarine did not inhibited RV infection (Lentz *et al*, 1997). Those results are in contrast to a reduction of infection in adult DRG neurons in culture after mecamylamine or D-tubocurarine treatment but not after α-BGT treatment (Castellanos *et al*, 1997). Then, IMR-32 could be expressing at low levels α-BGT-binding receptors that could account for partial inhibition of infection or the population of receptors that are mainly expressed varies with different culture conditions, which might explain different virus susceptibilities. It is not known yet which subunit combinations form functional receptors in sensory neurons, but differences in this matter between neuronal cell lines and primary cultures may account for differences in their susceptibility to viral infection and its pharmacological profile.

Thoulouze *et al* (1998) have demonstrated that NCAM is a receptor for a fibroblast-adapted rabies virus strain (CVS-BSR), because heparan sulfate (an endogen NCAM ligand), soluble NCAM, and antibodies to NCAM reduce infection in BSR and neuroblastoma cells. Additionally, the NCAM-deficient mice showed restricted brain invasion, especially to hippocampal and cerebellar neurons, precisely where there are high concentrations of nicotinic receptors. It has also been proposed that the low affinity neurotrophin receptor ($p75^{NTR}$) could act as rabies virus receptor, because fibroblasts transfected with the gene coding for this protein bind the viral glycoprotein and the street rabies virus itself with high affinity, suggesting an explanation for its high neurotropism (Tuffereau *et al*, 1998).

These studies in combination with our data suggest that neurons are infected not only through nicotinic receptors, but also using other molecules. A virus strain can use several receptors (i.e., NCAM or nAChR) probably because each of the elements of the quasispecies mix uses a specific receptor.

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