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Studying neurotrophin antiviral effect on rabies-infected dorsal root ganglia cultures

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Neurotrophin (NT)-induced modulation of rabies virus adsorption, transcription, and replication were analyzed in adult mouse dorsal root ganglia cultures. Different types of nerve growth factor and NT-3 treatment were tested before infection (pretreatment), during infection (transtreatment) and after withdrawing the viral inoculum (post-treatment). NT pretreatment for 4 days prior to infection produced a significant increase in the quantity of virus adsorbed into cultures and a concomitant increase in genomic viral RNA as measured by real time polymerase chain reaction (PCR). NT pretreatment triggered increased expression of two rabies virus receptors (NCAM and $p75^{NTR}$); however, no increase in rabies virus transcription and expression could be observed. By contrast, NT treatment during and after infection (trans- and post-treatment) induced a strong decrease in the quantity of viral nucleoprotein genomic and messenger nucleoprotein RNAs. These findings suggested that NT had an intrinsic inhibitory effect on rabies virus infection, which was not counterbalanced by NTs' rabies virus receptor–enhancing property and viral uptake. Adult mouse dorsal root ganglion cultures can be regarded as being a useful model for detecting therapeutic targets and evaluating experimental antiviral drugs. *Journal of NeuroVirology* (2005) **11,** 403–410.

Keywords: antiviral effect; neurotrophins; rabies; real time PCR; sensory neuron

Introduction

Rabies virus (RABV) (new abbreviation, following International Committee on Taxonomy of Viruses) is a highly neurotropic virus which can lead to lethal encephalitis. Sensory neurons present in dorsal root ganglia (DRG) represent one of the two routes for the virus making inroads into the central nervous system (CNS), capturing it during the initial stages of inoculation and transporting it towards the spinal cord (Murphy, 1977; Velandia *et al*, 2004). These neurons present neurotrophin (NT) receptors, such as nerve growth factor (NGF), Brain Derived Neurotrophic Factor (BDNF) and NT-3 receptors (Kashiba *et al*, 1995). NTs are small, structurally related proteins exercising many effects on the nervous system. A broad body of evidence has been assembled regarding NT effect on sensory ganglion neurons during both embryo (neurogenesis and proliferation) and adult stages (maintaining mature differentiated neuron phenotype) (Lindsay, 1992; Huang and Reichardt, 2001). NT acts through signaling cascades activated by tyrosine kinase, modifying many cell events (related to survival and differentiation); however, more subtle changes occur in adult mouse neurons, such as biochemical marker and neuropeptide expression

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(Lindsay, 1992; Mulderry, 1994; Kaplan and Miller, 2000). Evidence has been shown of changes produced by NGF affecting expression of $p75^{NTR}$ and NCAM (two molecules proposed as being RABV receptors). For example, NGF treatment induces a change in NCAM isoform expressed by rat pheochromocytoma PC12 cells (Prentice *et al*, 1987) and also modifies its yield in culture (Park *et al*, 1997). NGF treatment of PC12 produces a considerable increase in NCAM, in turn drastically increasing the proportion of RABV-infected cells (Thoulouze *et al*, 1998). Our group (Castellanos *et al*, 2000) has demonstrated that NGF and NT-3 modulate *in vitro* infection in sensory neurons. A significant reduction in the proportion of infected neurons was found by using NT concentrations of less than 0.5 nM, suggesting that NT may exert an antiviral effect *in vitro*.

The behavior of receptors for the virus ($p75^{NTR}$ and NCAM) on being treated with NT were analyzed in our cultures to elucidate how NT may exert their antiviral effect whilst up-regulating RABV receptor expression at the same time. Different types of treatment using NGF and NT-3 were tested in the present work, i.e., before infection (pretreatment), during infection (transtreatment), and after withdrawing the viral inoculum (post-treatment). We then evaluated NT effect on viral adsorption, on $p75^{NTR}$ and NCAM expression, and on viral transcription and replication activity. Two methods were used for evaluating infection. The first consisted of a classic immunocytochemical method for detecting cells infected 24 h post infection; the second method used real-time polymerase chain reaction (PCR) for quantifying viral genomic and nucleoprotein messenger RNA during an early postinfection period (6 h).

Results

Quantifying $p75^{NTR}$ and NCAM transcription and expression in NT-treated cultures

Real-time PCR was used for quantifying mRNA for two putative rabies virus receptors expressed in uninfected DRG cultures and its modification by NT exogenous administration for 4 days. A slight decrease (less than 50%) in the quantity of mRNA for $p75^{NTR}$ was found on being pretreated with NT (three independent experiments). There was no variation in NCAM messenger transcription in treated cultures.

A significant increase in $p75^{NTR}$ expression was found in ganglion cultures treated with 2 and 10 ng/ml NGF when a phosphorimaging system was used for quantifying protein expressed in treated cultures. A significant increase in NCAM expression was observed in NGF-treated cultures regarding control cultures (Figure 1). There was a slight no significant increase expression in $p75^{NTR}$ in NT-3–treated cultures. Two independent experiments were done in duplicate and then pooled.

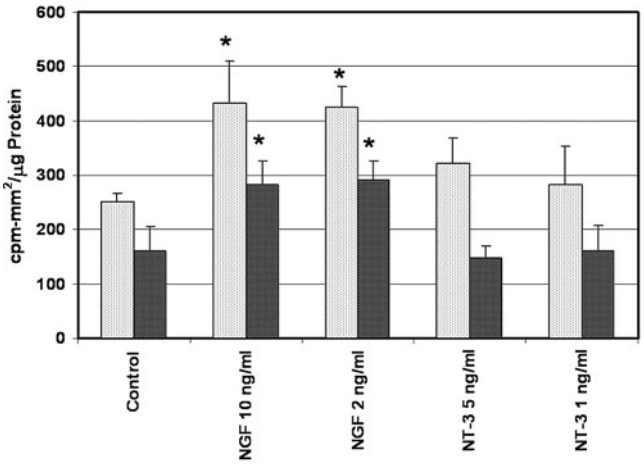

Figure 1 Quantifying NCAM (*clear bar*) and $p75^{NTR}$ (*dark bar*) expression in dorsal root ganglia cultures treated for 4 days with the aforementioned NT pretreatment. The data represent mean ± SEM of isotope signal corrected by each well's protein content. Asterisks indicate significant differences respecting untreated control ($P < .05$) after performing one-way ANOVA and LSD tests.

Quantifying RABV adsorbed into DRG cultures pretreated with NT

The quantity of viral particles bound to cultures during short incubation periods (10, 20, and 30 min) was measured for evaluating viral adsorption in normal DRG cultures and those treated with NT. Infected NT-treated cultures did not adsorb any more virus than nontreated infected ones following 10 min of incubation. It was found that both NGF and NT-3 treatment produced a significant increase in the quantity of virus adsorbed into the cultures following 20- and 30-min incubations. The quantity of absorbed viral antigen in NGF- or NT-3–treated cultures was significantly greater in all cases than that found in controls (Figure 2). Data represent the mean of two experiments done in duplicate.

Quantifying infection in DRG cultures

DRG cultures' main characteristic lies in their complex composition, consisting of neuron (30% of the total) and non-neuronal cells (70% Schwann cells and fibroblasts). This cell populations are easily recognizable: neurons are spherical, refringent, with evident growth cones and neurites, whereas fibroblast and Schwann cells are opaque flat cells with two or more lamellipodia. This characteristic led to it being confirmed that the virus has strong neuronotropism (χ^2 test, $P < .001$; $n = 12$) in this system as, in spite of neurons being in a minority in culture, 82% of infected cells were neurons (Table 1). Increasing the multiplicity of infection did not augment the percentage of infected non-neuronal cells but the percentage of infected neurons did increase by a third (rising from 30% to 39%, Table 1). It can be considered that non-neuronal cells are resistant to RABV infection (only 2% to 5%). No significant changes were

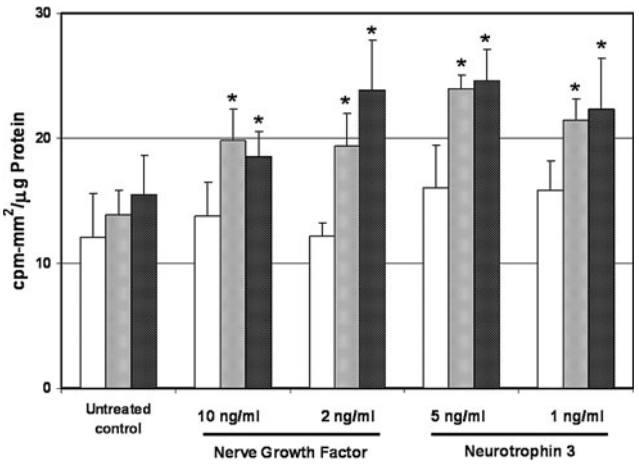

Figure 2 Quantifying rabies virus adsorbed at different times into NT-treated cultures for 4 days before infection (pretreatment), using a phosphorimager system. Adsorption times were 10 min (*clear bar*), 20 min (*gray bar*), and 30 min (*dark bar*). The data presented represent mean ± SEM, asterisks indicate significant differences respecting untreated control at the same time as evaluation ($P < .05$) using ANOVA and a *post hoc* LSD test.

found in the number of infected neurons following NT pretreatment when using the immunocytochemistry (ICC) technique; however, NGF (10 ng/ml) produced a blocking effect on being used concomitantly with the inoculum (transtreatment). NT-3 (1 ng/ml) produced a reduction in the number of infected neurons if administered after infection (post-treatment) (Table 2). Figure 3 presents images of rabies-infected DRG culture phase contrast and immunoperoxidase.

Modifying viral transcription and replication in NT treated DRG cultures

Quantities of genomic RNA (gRNA) and mRNA were measured during the viral cycle's initial period (6 h post infection) in NT-treated cultures for evaluating the step during which infection was being inhibited. The quantity of gRNA and mRNA for the nucleoprotein gene was expressed as being the ratio between the quantity of viral cDNA and the quantity of cDNA for β-actin in the respective sample. Modifications greater than 100% or less than 50% (respecting control) were considered to be valid. The data presented are the mean of three different experiments done in duplicate. Amplification products obtained during real time PCR were confirmed by separating them in ethidium bromide stained 1.2% agarose.

Table 1 Infected and noninfected cells of DRG cultures with two different MOI

	Noninfected	*Infected*	*Total*
MOI 0.1			
Neurons	1568 (60.2)	1035 (39.8)	2603 (100)
Non-neuronal	9968 (97.8)	222 (2.1)	10190 (100)
MOI 0.025			
Neurons	5388 (69.2)	2399 (30.8)	7787 (100)
Non-neuronal	15436 (94.6)	879 (5.3)	16315 (100)

Note. Data are cell number (percentage).

Table 2 Inhibition percentages of infected neurons respect to untreated infected cultures

	Inhibition percentage (±SEM)
Pretreatment	
NGF 2 mg/ml	1.6 ± 13.1
NGF 10 ng/ml	4.4 ± 7.8
NT-3 1 ng/ml	1.2 ± 8.1
NT-3 5 ng/ml	2.3 ± 6.8
Transtreatment	
NGF 2 mg/ml	6.8 ± 5.9
NGF 10 ng/ml	15.2 ± 3.7*
NT-3 1 ng/ml	8.2 ± 5.2
NT-3 5 ng/ml	4.9 ± 2.9
Post-treatment	
NGF 2 mg/ml	11.4 ± 6.7
NGF 10 ng/ml	3.8 ± 5.2
NT-3 1 ng/ml	17.8 ± 4.1*
NT-3 5 ng/ml	8.1 ± 5.3

Note. Data were drawn from immunocytochemical processed cultures and counts made in microscopy. Around 500 cells were counted in each coverslips, three experiments by quadruplicate were made ($n = 12$). Data are mean of inhibition percentage ± SEM. Asterisks indicate significant differences respect untreated infected control.

An increase greater than 100% (regarding untreated infected control) was found in all conditions when quantifying viral gRNA in cultures pretreated with NTs. However, no changes in transcription (viral mRNA) were presented in the same conditions. On the contrary, both neurotrophins treatment during infection (trans-) had significant reduction in mRNA nucleoprotein, but only NGF causes a fall in gRNA quantity. When neurotrophins were added after infection (post-treatment), cultures have ever less viral mRNA (less than 50% of the control) and less gRNA, except for the NGF 10 ng/ml (Figure 4).

In brief, when quantifying viral nucleoprotein mRNA, it was found that there was a strong reduction (70% to 94%) in the quantity respecting control in cultures treated with NT both during (trans-) and following (post-) infection, demonstrating a strong modification in viral transcription.

Discussion

NT treatment was applied at different times for ascertaining its role on RABV infection, using sensory neuron cultures. NT pretreatment increased NCAM and $p75^{NTR}$ expression without changing mRNA but did not induce changes in viral transcription or replication. Trans- and post-treatment caused a marked decrease in the quantity of viral nucleoprotein genomic and messenger RNA, suggesting an inhibitory role for NT in viral transcriptional/replicative events.

Increased quantities of gRNA and virus becoming adsorbed in pretreated cultures could have been be

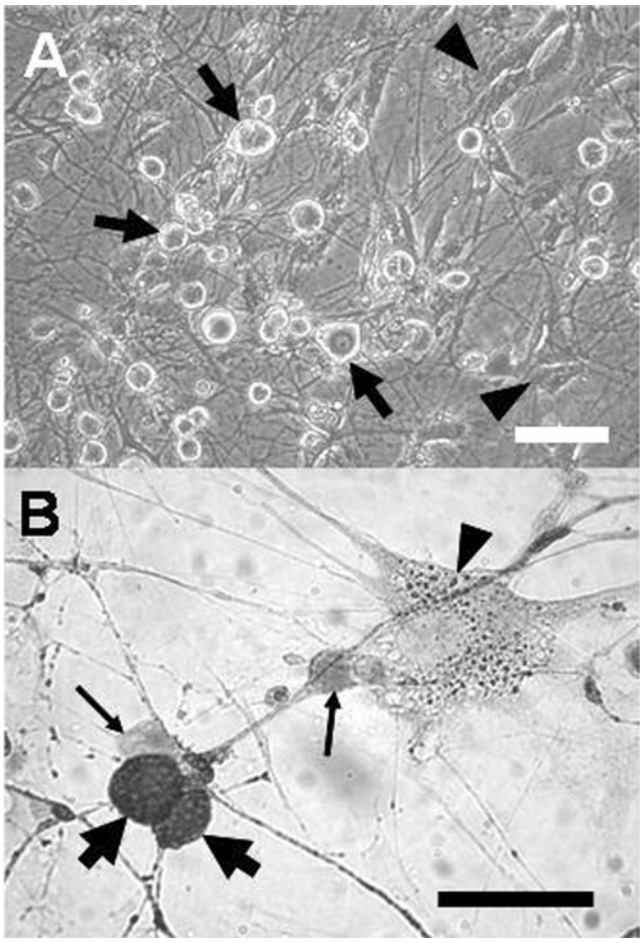

Figure 3 **A**, Adult mouse dorsal root ganglion cultures contain several types of cells: neurons (*arrows*) and non-neuronal cells (fibroblasts and Schwann cells; *arrow head*). Neuron to non-neuronal cell proportion was 1:3; however, most cells infected by rabies virus were neurons. **B**, Indirect peroxidase immunostaining of infected cells. Note the difference in the aspect of infected neurons, they are spherical with neuritic tree (*thick arrow*) and positive non-neuron cells, which are large, flat, and fibroblast-like (*arrow head*). The thin arrows indicate uninfected neurons. Bar correspond 100 μm.

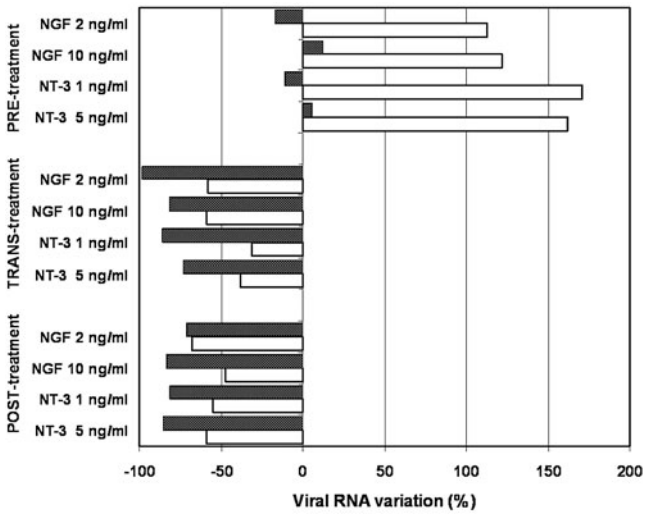

Figure 4 Percentage of viral RNA quantity changes induced by NT in dorsal root ganglia infected cultures respecting untreated ones. Genomic RNA (*clear bar*) and nucleoprotein mRNA (*dark bar*). NT treatment was applied 4 days before infection with the virus (pretreatment). The viral inoculum was prepared in culture medium with each NT (transtreatment) or added after withdrawing and washing viral inoculum (post-treatment). Data represent the mean of three independent experiments processed by duplicate. Increases greater than 100% and reductions greater 50% were felt to be significant.

related to three findings. There was increased neurite extension (larger virus capturing network), being 6.3% and 42.9% for 2 and 10 ng/ml NGF and 14.8% and 13.7% for 1 and 5 ng/ml NT-3, respectively. Secondly, NGF promoted medium- and large-sized neuron survival as compared to untreated controls (unpublished results), therefore leading to an increase in these neurons being more susceptible to infection, as has been demonstrated in other works (Castellanos *et al*, 2000; Martínez-Gutierrez *et al*, 2002). Thirdly, there was putative RABV receptor up-regulation.

NCAM has been shown to play an important role in adsorption and the spread of rabies virus (Thoulouze *et al*, 1998). $p75^{NTR}$ participation has also been reported in wild strain virus NGF (Tuffereau *et al*, 1998, 2001). $p75^{NTR}$ is mainly expressed during development. It is expressed in adults following axonal injury or in pathological situations such as epilepsy and neurodegeneration. Despite $p75^{NTR}$ having been detected in some neuron populations' axons and cell bodies in adult rats' CNS (Pioro and Cuello, 1990a, 1990b; Dougherty and Milner, 1999), it is not expressed in spinal cord motoneurons in motor nerve endplates or at terminal intramuscular axon branches (Sheard*et al*, 2002; Copray*et al*, 2003). It is essentially seen in the dorsal horn in adult rats' spinal cords and more rarely in the ventral horn (Pioro and Cuello, 1990b).

We found that NGF pretreatment increased the quantity of $p75^{NTR}$ and NCAM expressed by culture without a significant change in the quantity of mRNA transcripts, indicating a probable role for NGF regulating turnover for this protein. It should be stressed that, according to preliminary results from our laboratory, both $p75^{NTR}$ and NCAM are expressed in at least 50% of cultured neurons and a small proportion of cultured non-neuron cells (fibroblasts and Schwann cells) (data not shown).

This finding could be contradictory, because it would be supposed that all cultured cells should be susceptible to infection; however, only 2% to 5% non-neuronal cells were infected, which can be considered to be very low (within the level for refractory cells becoming infected) possibly due to NCAM isoform being expressed by these cells. Taking this protein's presence in all culture cells into account, it became necessary to evaluate how (whether) NGF treatment modified $p75^{NTR}$ or NCAM expression.

Reduced viral nucleoprotein mRNA in NT-treated culture could have been related to modulating some

enzyme route implicated in activating L-P-N complex transcriptional function (possibly kinase or phosphatase, see below). A competition effect (i.e., with $p75^{NTR}$, on preparing inoculum with NT) may possibly have been happening but a fall in virus transcription activity was also produced when treatment was applied following infection. Both NT treatments led to reduced gRNA in culture; viral receptor competition could therefore be considered, but a net antireplication effect should also not be discarded. The high percentage of messenger reduction (up to 94%) could mean that primary activity was transcriptional during early postinfection evaluation (6 h), therefore more efficiently blocking the replication activity, which must happen during later stages of the viral cycle.

Rhabdovirus transcriptional activity depends on the correct status of P and N phosphorylation which, together with the polymerase (L), uses the RNA template associated with N (RNA-N) to produce messengers. A consensus suggests that rabdovirus needs viral protein cell kinase. For example, it is well-known that casein kinase II participates in rabies N and VSV P phosphorylation (Wu *et al*, 2002, 2003) and protein kinase C (PKC) in RABV and human parainfluenza virus phosphorylation (Gupta *et al*, 2000; De *et al*, 1995).

It is suggested that P phosphorylation and dephosphorylation could be responsible for L-P or N-P complex changes in affinity and formation and consequent switch of transcriptional-replication activity. VSV and RABV phosphoprotein phosphorylation confers two or more forms on them, having different electrophoresis migration patterns (Takamatsu *et al*, 1998) and presumably having different functional states as those viruses possessing few genes employ this strategy so that proteins may fulfill many functions (Lenard, 1999).

It has been found that PKC and another enzyme from mouse brain (designated rabies virus protein kinase, RVPK) very efficiently phosphorylate rabies virus phosphoprotein *in vitro* (Gupta *et al*, 2000); it has been proposed that this particular nervous system enzyme could be playing a key role in the development of nervous pathogeny. P phosphorylation by PKC or RVPK causes severe modifications in secondary structure. RVPK activity causes P to migrate more slowly (40 kDa), whilst PKC does not change migration (36 kDa). There has been speculation about the role played by each isoform in transcriptional or replication events, also in changing affinity for L or N as has been demonstrated for VSV.

Due to these precedents, it can be suggested that the NT antiviral effect found in this work may have been related to activating serine-theronine kinase phosphorylating P in a domain inhibiting transcription or inducing the appearance of a P isoform, drastically changing its activity. It could also be related to activating phosphatase, thus eliminating the P or N phosphate group and preventing transcriptase activity. The effect of NT does not seem to have been due to regulating NT-induced gene expression, because evaluation was done very shortly post infection, even though it cannot be discarded that transcription factor phosphorylation induces cell kinase expression (for virus phosphorylation) or that NT-induced Ca^{2+} release produces the antitranscriptional effect.

This work stated that there are specific neuronal pathways involved in rabies infection regulation, therefore it open the way forward for research using the rabies virus because the cellular factors responsible for the viral replication cycle can be directly determined from the model used. Possible therapeutic targets can thus be evaluated when designing drugs for use in an infection which, once it reaches the central nervous system, inevitably leads to death. Our group's standardized sensory neuron interaction-infection model in culture has proved to be an excellent tool for basic investigation regarding the different events in the rabies virus replication cycle.

Materials and methods

Dorsal root ganglion cell culture

Procedures used in this work were approved by the Colombian Instituto Nacional de Salud's (INS) Ethics Committee. ICR adult mice (25 g) were sacrificed by cervical dislocation; the vertebral column was obtained by separating it from the ribs, and cervical and sacra area with surgical scissors in sterile conditions. It was then divided into two halves (ventral and dorsal) and DRG were located and extracted using a stereoscope. They were then placed into a collagenase solution (200 U/ml; Gibco BRL), incubated for 90 min, completing dissociation by repeatedly passing the cell suspension through a narrow-end Pasteur pipette (Castellanos and Hurtado, 1999). A total of 40,000 viable neurons from this suspension were sown in each 35-mm diameter dish for molecular biology (MB) experiments and 2000 viable neurons/well were seeded on round glass coverslips in 24-well dishes for counting infected cell number by ICC. Petri dishes and coverslips were pretreated with 10 μg/ml poly-L-lysine. Culture medium change was first changed after 48 h, adding 10 μM cytosine β-arabinofuranoside (Sigma-Aldrich); cells were kept at 37°C with 5% CO_2. Dulbecco's modified Eagle's medium (DMEM) was used plus 10% fetal bovine serum (FBS), 100 U/ml penicillin, and streptomycin (100 μg/ml) (complete culture medium, CCM) which was changed each 48 h, adding the respective NT (see later).

Neurotrophin treatment

The NT used were 2 and 10 ng/ml NGF (Gibco BRL) and NT-3 (1 and 5 ng/ml; Promega), prepared in CCM, which had previously been found to be inhibitory (Castellanos et al, 2000). Three NT treatment schemes were used at different times during culture. The first

treatment was begun when the cells were sown; NT were again added at the first change of medium after 48 h and discarded 3 hours before infection (pretreatment: 4 days total time). The second treatment was only applied during infection; the viral inoculum was prepared in the corresponding NT concentration and added to cultures (transtreatment: 1 h total time). The third treatment consisted of NT being added to the culture immediately after withdrawing the viral inoculum (post-treatment) and being kept for 6 (MB experiments) or 24 h (ICC experiments).

Virus, culture infection, immunocytochemistry, and infected cell count

CVS-11 (challenge virus standard) virus donated by the INS Anti-rabies Vaccine Production Laboratory (PAHO/WHO reference virus) was replicated in ICR adult mouse brain. A 20% suspension of homogenized cerebral tissue (prepared in phosphate-buffered saline [PBS] with 2% horse serum) was put into aliquots and kept in liquid nitrogen until use. The virus was titered by intra-cerebrally inoculating groups of 10 young mice with seried dilutions. The dose producing death in 50% of the animals was evaluated; a $10^{7.31}$ DL_{50} viral titer was obtained.

The cells were infected for 1 hour with a virus stock dilution prepared in CCM. ICC was used for evaluating multiplicity of infection (MOI) in two viral dilutions (0.1 MOI and 0.025 MOI); these low viral dilutions were used for promoting specific interaction with the most susceptible cells. Cultures for MB experiments were infected with 0.1 MOI. The inoculum was withdrawn, cells were washed with CCM, and incubated for different times, depending on the type of experiment (6 h for MB and 24 h for ICC).

Cultures were fixed for 30 min with 4% paraformaldehyde prepared in PBS for infected cell count. They were then permeabilized with 0.1% Triton X-100 for 30 min. Endogenous peroxidase was quenched with a 0.3% H_2O_2 solution prepared in 10% methanol in PBS, washed, and incubated with blocking buffer (BB; PBS plus 10% horse serum). Anti-rabies antibody (anti-whole virus produced in hamster at the NIH Colombian reference antibody) was added for 30 min in antibody buffer (0.05 M Tris, 0.5 M NaCl, 0.1% Triton X-100, pH 8.6). After being washed, biotinylated anti-hamster prepared in BB (2.5 μg/ml; Vector Labs) was added for 30 min and then incubated with avidin and biotinylated peroxidase complex (Vector Labs). This was revealed with a 0.02% H_2O_2 and 0.075% diaminobenzidine solution prepared in Tris-HCl, pH 7.2. Cultures were dehydrated in successive ethanol baths; glass coverslips were then mounted with Poly-Mount (Polysciences). Five hundred cells, and also the number of infected cells (including neurons and non-neuronal cells), were counted in random fields for each glass coverslip by microscope (Olympus BX 50) at 400× magnification (Castañeda-Castellanos *et al*, 2002). Three cultures were prepared, having four replicas per condition, and data were then grouped ($n = 12$). Angular transformation was made from the proportion of infection; data were analyzed with one-way analysis of variance (ANOVA), followed by Newman-Keuls mean multiple comparison test.

DRG culture total RNA purification, RT-PCR for viral mRNA, genomic RNA, β-actin, $p75^{NTR}$, and NCAM mRNAs

Total RNA was extracted from infected and noninfected cultures for the MB experiments using the guanidine/chloroform thiocyanate method (Trizol; GIBCO BRL). RNA was suspended in RNase-free water (treated with 0.1% DEPC) and quantified by spectrophotometry. Reverse transcription was done from 1.5μg total purified RNA using an Oligo-dT(15) primer (Promega) and AMV reverse transcriptase (Boehringer Mannheim) for mRNA. A specific viral nucleoprotein gene (5′-GGAATTCTCCGGAAGAC TGGACCAGCTATGG-3′) positive-sense primer was used for viral genome cDNA synthesis, using the same reverse transcriptase.

Various conditions were assayed for optimizing PCR for each gene to be used. PCR was standardized for a segment of the rabies virus N-CVS-F 5′-GGA-ATTCTCCGGAAGACTGGACCAGCTATGG-3′ nucleoprotein gene (CVS strain) and the N-CVS-R 5′-AG-AATTCCCACTCAAGCCTAGTGAACGC-3′ primer. The pRevTRE plasmid (Clontech) into which the complete gene for the CVS nucleoprotein was inserted was kindly donated by Dr. Christophe Préhaud from the Viral Neuroimmunology Unit (Pasteur Institute, Paris). Raid Kassis from the Pasteur Institute Rabies Unit (Paris) constructed the plasmid with the gene for β-actin. M1 5′-ATCCTGTTG CTCCCTGGAGA-3′ and M2 5′-TGCCTGGGTACAT GGTGGTA-3′ primers were used for amplifying a segment from the β-actin gene. p75-11 5′-TATAGACTC CTTTACCCACG-3′ and p75-12 5′-AATGTCAGCTC TTGGATGC-3′ primers and plasmid with $p75^{NTR}$ gene (pMV7) were used for p75 PCR. Mars1 5′-CCAGCAGCGGATCTCAGTGGTGTGG-3′ and Mars3 5′-GATGGTTGGAGGCAGGGAGCTGACC-3′ primers and pRC/CMV plasmid with the gene encoding 180 kDa NCAM were used for NCAM amplification, a kind gift from Harold Cremer (Cremer *et al*, 1994).

Real-time PCR relative quantification of viral messenger and genomic RNA in infected cultures

Viral transcription and replication were quantified in both NT-treated and untreated infected cultures using a real-time PCR system allowing starting cDNA to be quantified in each sample, extrapolating it in a standard amplification curve (processed simultaneously), using known concentrations of the same gene previously cloned in plasmid. Primers and plasmid used for quantification were those reported in the previous paragraph. The number of β-actin molecules in each samples were quantified by using

the same system to normalize data from each amplified product regarding a housekeeping gene. An ABI Prism 7700 Sequence Detection System (Perkin-Elmer Biosystems) was used for real time amplification and analysis. mRNA was quantified for two putative RABV receptors ($p75^{NTR}$ and NCAM) in NT-treated cultures. Data were always reported as receptor cDNA/β-actin cDNA or viral cDNA/β-actin cDNA ratio and expressed as control percentage variation.

Phosphoimager analysis: RABV binding and $p75^{NTR}$ and NCAM expression

DRG cells were plated on poly-L-lysine treated eight-well slides (Labtek; Becton Dickinson) for evaluating virus adsorption in NT-pretreated cultures for 4 days. A viral suspension (0.1 MOI) was prepared and added to the cells, leaving them in contact for 10, 20, and 30 min at 4°C. The respective wells were washed and fixed with 4% paraformaldehyde for 20 min at the end of each time. They were then incubated with a monoclonal antibody (PVA3) directed against the viral N protein, followed by incubation with I^{125}-coupled goat anti-mouse immunoglobulin (IgG) (1 μCi/well; Amersham Pharmacia Biotech). Slides were analyzed in a high-resolution β-imager (Biospace, France). The same procedure was adopted for NT-treated (or untreated) cultures, but using monoclonal anti-NCAM (Pharmingen) and an anti-$p75^{NTR}$ polyclonal antibody (Promega).

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