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Effects on synaptic activity in cultured hippocampal neurons by influenza A viral proteins

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Certain viruses can infect neurons and cause persistent infections with restricted expression of viral proteins. To study the consequences of such viral proteins on synaptic functions, the effects of two influenza A virus proteins, the nonstructural protein 1 (NS1) and the nucleoprotein (NP), were analyzed in cultures of rat hippocampal neurons. Transduction of the NS1 and NP proteins into the neurons was performed by applying the 11–amino acid peptide transduction domain (PTD) of human immunodeficiency virus (HIV) TAT coupled to the viral proteins. Neurons exposed to the NS1 and NP fusion proteins (NS1-PTD and NP-PTD, respectively) for 4 h were immunopositive for these proteins as diffuse cytoplasmic and nuclear distribution. After exposure for 48 h to NP-PTD, a punctate pattern of the immunolabel appeared in dendritic spinelike processes. Electrophysiologically, a reduction in both the frequency of spontaneous excitatory synaptic activity and in the amplitude of the miniature excitatory postsynaptic currents were recorded after exposing the hippocampal neurons to NP-PTD between 17 and 22 days in culture. These changes may reflect disturbances in postsynaptic functions. No such alterations in synaptic activities were recorded after exposure to NS1-PTD or to green fluorescent protein–PTD, which was used as a control. Based on these findings the authors hypothesize that the viral NP, by its localization to dendritic spinelike structures, interferes with the expression or anchoring of postsynaptic glutamate receptors and thereby disturbs synaptic functions. Thus a persistent viral infection in the brain may be associated with functional disturbances at the synaptic level. *Journal of NeuroVirology* (2005) **11,** 395–402.

Keywords: brain; nervous system; neuron; peptide transduction; synapse; viral infection

Introduction

Several viruses can spread to the nervous system and infect different populations of neuronal cells. Because the nervous system provides an immunopriviledged site, such infections may not be readily cleared. Because of this, many viruses can persist in the nervous system for extended periods of time. RNA viruses, for example, may persist at this location in a state with restricted synthesis of the different viral proteins (Kristensson and Norrby, 1986; Oldstone, 1989; Tyler and Nathanson, 2001). This fact poses the question whether the presence of viral components in a neuron can cause changes in its function and, in particular, in its synaptic transmission. Here, we have chosen the neurotropic influenza A/WSN/33 viral strain, which infects neurons in the substantia nigra and the hippocampus and causes a lethal infection following intracerebral or systemic injections in mice (Takahashi *et al*, 1995), but a nonlethal infection targeted to monoaminergic neurons in the midbrain after injection into the olfactory bulbs (Mori *et al*, 1999). The latter infection is associated with long-lasting alterations in expression of synaptic

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regulatory genes combined with changes in cognitive and emotional behaviors (Beraki *et al*, 2005). Human pathogenic influenza A virus strains may occasionally be neurovirulent as recently illustrated by the Hong Kong 1997 epidemics from which viruses with neurotropic properties in mice, ferrets, and primates were isolated (Park *et al*, 2002; Rimmelzwaan *et al*, 2001; Zitzow *et al*, 2002). Furthermore, maternal influenza A virus infections have been associated in some but not all studies, with an elevated risk for the offspring to develop psychiatric diseases later in life (for review, see Munk-Jorgensen and Ewald, 2001).

Although influenza A virus does not, in general, persist in mammals (Doherty *et al*, 1996), more than a year after its injection into the olfactory bulbs, viral RNA has been detected by reverse transcriptase–polymerase chain reaction (RT-PCR) in immunodefective mice with targeted disruption of the transporter associated with antigen presentation 1 (TAP1) gene (Aronsson *et al*, 2001). In particular, RNA of the nonstructural 1 (NS1) protein and the nucleoprotein (NP) have been found in sections taken at midbrain levels in almost all such mice (Aronsson *et al*, 2001). Furthermore, in offspring of wild-type mice infected intranasally with the WSN/33 strain at day 14 of pregnancy, RNA encoding the viral NP or matrix 1 (M1) proteins has been found in a portion of the brains at postnatal day 90 (Aronsson *et al*, 2002).

In cultures of rat embryonic hippocampal neurons, we previously observed that the WSN/33 strain caused an infection accompanied by a reduction in voltage-dependent calcium currents (Brask *et al*, 2001). In such neurons NP immunolabeling occurs in the nuclei and somatodendritic domains as well as colocalizing with α-actinin in dendritic spinelike structures, which was prominent after overexpression of NP introduced using the Semliki Forest virus vector system (Owe-Larsson *et al*, 1999). This vector system only permits short-term observations and to allow long-term analysis of effects of individual viral proteins, we employed here a system of protein transduction using the peptide transfer domain (PTD) of the human immunodeficiency virus (HIV) TAT protein (Frankel and Pabo, 1988; Green and Loewenstein, 1988). The unique property of PTD is its ability to transduce proteins through the plasma membrane into the cytoplasm and nucleus of a cell after extracellular application. The PTD is an 11–amino acid long peptide that does not include the toxic part of the TAT protein (Frankel and Pabo, 1988; Green and Loewenstein, 1988). It has been well documented that PTD can transport a variety of proteins with retained bioactivities into cells both *in vitro* and *in vivo* (Wadia and Dowdy, 2003). We have coupled the genes encoding PTD with those of the viral proteins and exposed hippocampal neurons in culture to the gene products (NP-PTD and NS1-PTD).

We here report that NP-PTD localizes to dendritic spinelike structures in the neurons after long-term exposure, resulting in a reduction in spontaneous excitatory postsynaptic currents (sEPSCs) and a decrease in amplitude of the miniature excitatory postsynaptic currents (mEPSCs).

Results

Transduction of NS1-PTD into N1E-115 cells

The transduction into N1E-115 cells of three different constructs in which NS1 coupled to the PTD (Figure 1) was examined. In one construct, the PTD was inserted in front of the NS1 gene and, in another, behind the gene. However, none of the proteins expressed from these two constructs were transduced into N1E-115 cells as revealed by immunofluorescence. The N1E-115 cells were, however, immunolabeled after exposure to the third construct, in which four glycines had been inserted between the PTD and NS1 gene to create a space between the PTD and the NS1 protein for increasing the chance of exposure to the PTD epitope. This successfully transduced construct is referred to as NS1-PTD in the following.

Transduction of the NS1-PTD and GFP-PTD proteins into hippocampal neurons

Hippocampal neurons kept for 17 days in culture (DIC) were exposed to various concentration of the NS1-PTD construct (Figure 1). A green fluorescent protein (GFP)-PTD construct with four-glycine inserts was used as a control. When hippocampal neurons were exposed to NS1-PTD at a concentration of 0.5 and 0.75 μM for 4 h, 5% and 35% of them became immunopositive, respectively, and 85% to 95% of the cells were immunopositive when exposed to concentrations of 1.5, 2.25, and 4.5 μM. The immunolabeling

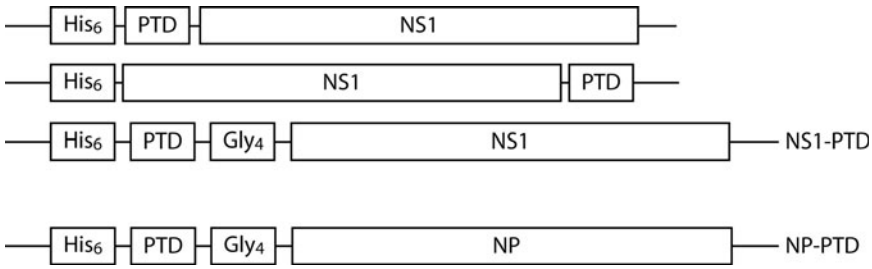

Figure 1 Schematic drawings of the three NS1-PTD constructs and the NP-PTD construct.

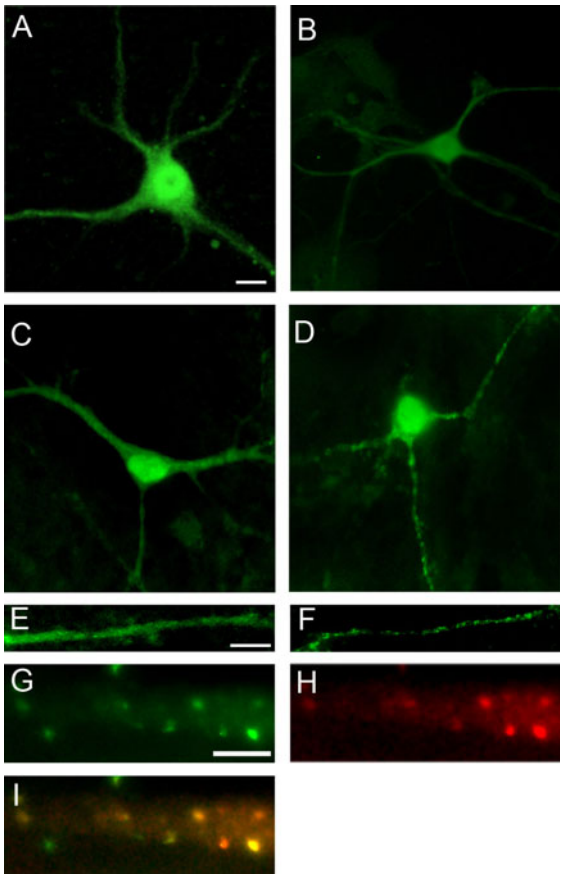

Figure 2 Immmunofluorescence showing the presence of viral proteins in hippocampal neurons exposed to NS1-PTD for 4 h (**A**), GFP-PTD (**B**), and to NP-PTD for 4 h (**C**) and 48 h (**D**). Note the punctated immunolabeling of NP in the dendritic-like processes and after 48 h (**F**) compared to after a 4-h (**E**) exposure. (**G–I**) Double immunolabeling of dendritic spine-like structure with antibodies to NP (*green*, **G**) and α-actinin (*red*, **H**); merged (**I**). Scale bars 10 μM (**A–I**), 5 μM (**G–I**).

was diffusely distributed in both the soma and the nuclei (Figure 2A). A similar distribution of the GFP-PTD construct was seen (Figure 2B). The NS1-PTD and GFP-PTD constructs had no toxic effects at concentrations of 0.5 to 1.5 μM, whereas 2.25 μM of the proteins caused a nerve cell loss of 23% and 31%, respectively, after 7 days of exposure. Exposure to 4.5 μM of the two proteins caused a nerve cell loss of 71% and 78%, respectively. Because the nerve cell loss occurred to a similar extent for the two constructs, this may reflect a toxicity of the PTD part of the construct.

Transduction of the NP-PTD protein into primary hippocampal neurons

About 35% of the hippocampal neurons (17 DIC) were immunopositive for NP after exposure for 4 h to 0.25 μM of the NP-PTD protein with four-glycine inserts between the PTD and NP. After exposure to 0.5, 0.75, or 1 μM, about 85% to 90% of the neurons were immunopositive. The NP immunostaining was diffuse and intense in the nuclei, but weaker in the somatodendritic domains, after 4 h of exposure to 0.5 μM of the construct (Figure 2C and E), whereas after 48 h of exposure a punctate immunolabeling was observed in the dendrite-like processes (Figure 2D and F). The NP immunostaining overlapped with that of α-actinin (Figure 2G–I). No nerve cell loss was seen after exposure for 7 days to this concentration, whereas exposure to 1.0 and 1.5 μM of the construct caused a loss of about 18% and 55% of the neurons, respectively, within 7 days.

Exposure to NP, but not NS1, reduced the spontaneous excitatory postsynaptic activity in hippocampal neurons

Neurons exposed to NP-PTD at a concentration of 0.5 μM between 17 and 22 DIC and recorded at 25 DIC showed a significant reduction in the frequency of spontaneous excitatory synaptic activity (Figure 3A), yet inhibitory synaptic activity was unaltered (Figure 3B). No such alterations could be detected in the spontaneous synaptic activities of cultures exposed to either the NS1-PTD or the GFP-PTD at a concentration of 1.5 μM between 17 and 22 DIC and recorded at 25 DIC.

NP-PTD reduced the amplitude, but not the frequency, of mEPSCs

To analyze whether the observed reduction in excitatory synaptic activity reflected changes at pre- or postsynaptic sites, we measured the frequency and the amplitude of mEPSCs in the presence of 1 μM Tetrado-toxin (TTX), which blocks the firing of action potentials and, consequently, spontaneous synaptic transmission. In the NP-PTD–exposed cultures, a significant reduction in the amplitude of mEPSCs was observed, but there were no significant changes in their frequency as recorded at 25 DIC after exposure to NP-PTD between 17 and 22 DIC (Figure 4).

Discussion

The recent discovery that certain proteins can be transduced across cell membranes of eukaryotic cells has led to the identification of PTDs. Examples of these proteins are the *Drosophila* homeoprotein (antennapedia transcription protein; Ant HD), the Herpes simplex virus structural protein VP22, and the HIV transcriptional activator TAT protein (Joliot and Prochiantz, 2004; Prochiantz, 2000). Such proteins or PTDs can be coupled to other proteins for their transduction across plasma membranes into the cytosol and nucleus of a cell (Wadia and Dowdy, 2003). The HIV TAT has an 11–amino acid PTD that mediates the transduction. The mechanism for transduction of the peptide across the lipid bilayer is still not clearly understood, although this process is clearly not mediated by a receptor (Fittipaldi *et al*, 2003; Mi *et al*, 2000). Recently, the positively charged TAT PTD was found to interact with negatively charged components of the cell membrane,

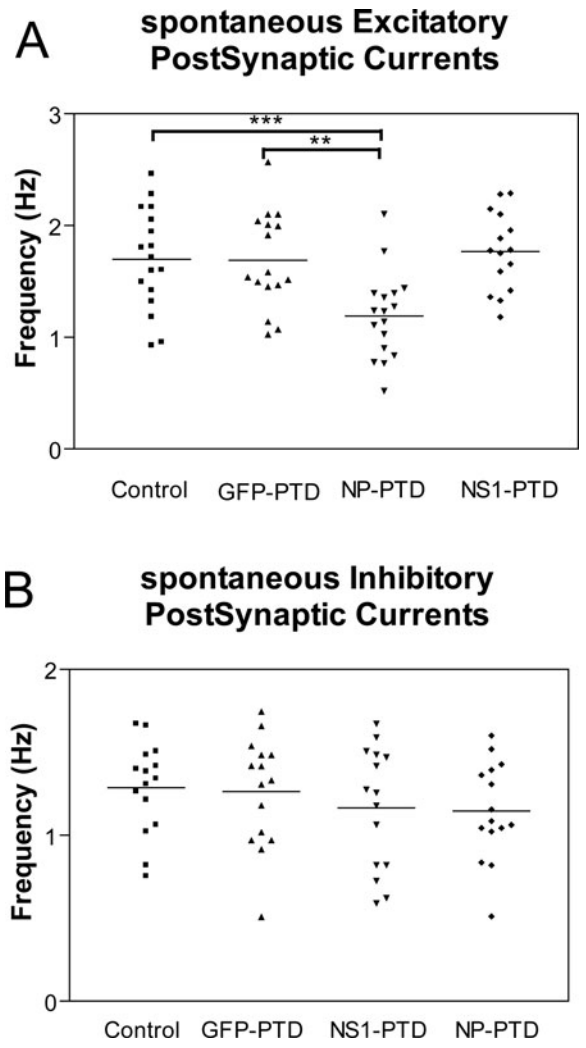

Figure 3 Comparison of the frequency of sEPSCs and sIPSCs between controls and cultures exposed to GFP-PDT, NP-PTD, and NS1-PTD between 17 and 22 DIC and recorded at 25 DIC. (**A**) The sEPSCs were significantly reduced in cultures exposed to NP-PTD compared to untreated control cultures and to cultures exposed to GFP-PTD. Cultures exposed to NS1-PTD and GFP-PTD did not significant altered the sEPSCs activity compared to untreated control. $^{**}P > .01$; $^{***}P > .001$ (**B**) No significant changes in the sIPSCs could be detected among the groups.

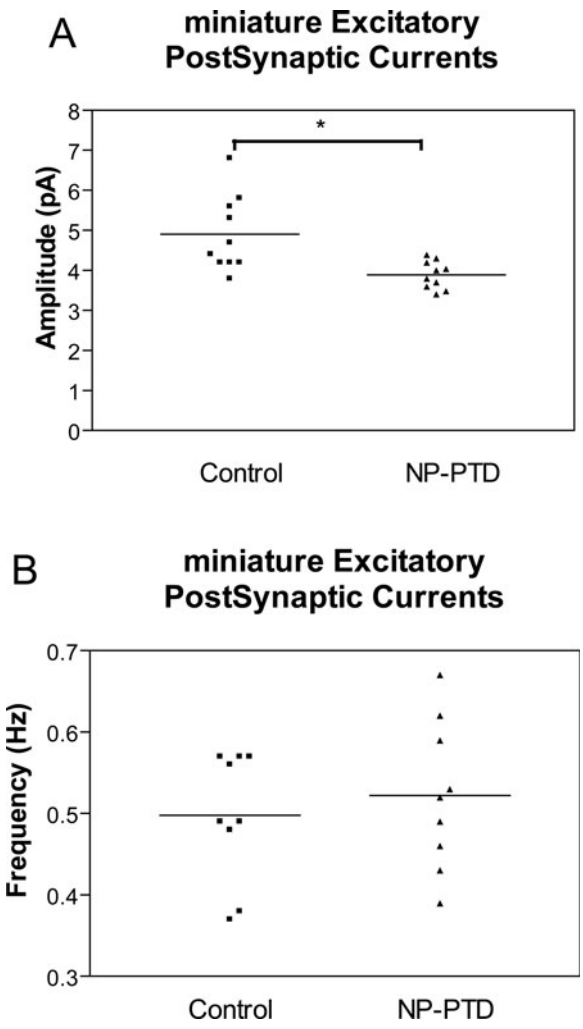

Figure 4 Comparison of the frequency and amplitude of mEPSCs in controls and cultures exposed to NP-PTD during 17–22 DIC. (**A**) The amplitudes of mEPSCs were significantly reduced in NP-PTD-exposed cultures.$^{*}P > .05$. (**B**) The mEPSCs frequencies were not significantly different in controls and neurons exposed to NP-PTD at 17 to 22 DIC and recorded at 25 DIC.

causing membrane inversion during transduction. This indicates that the transduction across the membrane involves membrane lipid movement and rearrangement (Del Graiza Moore and Rayne, 2004).

A number of studies have successfully adopted the HIV TAT PTD system, and each of at least 23 different TAT PTD fusion proteins described to date retain their biological activities when transduced into different cell types (Wadia and Dowdy, 2003). The TAT PTD contains the nuclear localization signal of the TAT protein (Vives *et al*, 2003). Although the TAT PTD system provides a powerful technique to transduce proteins into cells, it involves a number of problems. One major difficulty is that the purification step using His-tagged proteins results in denatured products. These denatured proteins, once inside a cell may, or may not, be correctly refolded by chaperones (Gottesman *et al*, 1997). Proteins prepared by this method may also have a greater biological effect than their counterpart prepared in soluble conditions (Nagahara *et al*, 1998). Studies using this technique on nerve cells are hitherto few. However, one such example is the transduction of a functional domain of the angiotensin II type I receptor into neuronal cultures from rat hypothalamus and brainstem, which led to an increased firing rate of spontaneous action potentials (Vazquez *et al*, 2003).

The influenza A NS1 is synthesized in infected cells, but is not incorporated into virions. It is a phosphoprotein that accumulates in the nucleus and, at later stages of infection, also in the cytoplasm of an infected cell (Lazarowitz *et al*, 1971). NS1 is involved in the inhibition of production of antiviral

interferons α/β in infected cells, and it exerts these activities as a dimer (Krug *et al*, 2003). NS1 expressed in Madin-Darby Canine Kidney (MDCK) or HeLa cells can also induce apoptosis, but it is not clear whether or not dimerization of NS1 is required for this process (Schultz-Cherry *et al*, 2001). In addition, NS1 may during an infection have interferon-dependent, antiapoptotic potentials (Zhirnov *et al*, 2002). In the present study, the NS1-PTD caused no morphological structural changes in the neurons and no signs of apoptosis were seen. We do not know if this lack of effects is due to methodological problems or not. The NS1-PTD construct was therefore only used as a control, in addition to the inert GFP-PTD, for comparison with the NP-PTD in the electrophysiological studies.

NP is the major component of the influenza ribonucleoprotein (RNP) particle. Early in infection, NP is imported to the nucleus through the nuclear pore after its nuclear localization signal has bound to the importin α receptor. This event is followed by export of the protein back to the cytosol after binding to the nuclear export receptor CRM1. This translocation to the cytoplasm is dependent on phosphorylation of NP (Bui *et al*, 2002). In addition to binding single-stranded RNA, importin α, and CRM1, NP has been found to bind tightly to F-actin, and this interaction between NP and F-actin may play a role in the retention of the RNP in the cytoplasm at later stages of infection (Digard *et al*, 1999). After a 48-h exposure to the NP-PTD, we noted a marked pattern of punctate immunostaining in the dendritic-like processes. This pattern is similar to that previously observed after overexpression of NP using the Semliki Forest virus expression system, in which NP colocalized to α-actinin (Brask *et al*, 2001). Because α-actinin localizes to, and can be a marker for, dendritic spines, it is likely that the NP accumulates over time in these structures. Considering the role of actin and actin-binding proteins in determining anchoring and expression of glutamate receptors at postsynaptic sites (Tashiro and Yuste, 2003; Kasai *et al,* 2003), it could be speculated that the actin-binding NP may alter the physiological properties of the postsynaptic spines.

In support of the notion that NP may cause postsynaptic changes, we found electrophysiologically a reduction in frequency of the sEPSCs in cultures exposed to NP-PTD between 17 and 22 DIC. The presently observed changes are most likely related to effects of the NP rather than to the TAT-PTD constructs, because neither the GFP-PTD nor NS1-PTD constructs caused any electrophysiological changes in the culture, although the cultures were exposed to even higher concentrations of these constructs. Because the amplitude, but not the frequency, of the mEPSCs was reduced, disturbances in postsynaptic functions most likely underlie the observed reduction in sEPSCs activity. We therefore hypothesize that, by binding to actin and localizing in dendritic spinelike structures, the viral NP protein may interfere with excitatory synaptic functions.

In conclusion, our study shows that proteins can be transduced into the cytosol as well as the nucleus of neurons using TAT PTD, and that the viral NP may localize to dendritic spinelike structures after long-term exposure to cause alterations in postsynaptic functions. This process, if present *in vivo,* could provide a potential pathogenic mechanism by which dysfunctions may arise in neurons that harbor persistent viral infections in the brain.

Materials and methods

Virus culture and virus titration

Influenza virus A/WSN/33 (H1N1) and the hybrid influenza A virus R404BP strains were obtained from Dr. S. Nakajima (The Institute of Public Health, Tokyo, Japan). They were grown in MDCK cells maintained in Dulbecco's modified Eagle's medium (DMEM), 2 mM L-glutamine, and 0.2% bovine serum albumin (BSA) (all from Sigma Chemical, St Louis, MO, USA) and incubated for 24 h. The supernatants were titrated by plaque assay on MDCK cells and stored at -70°C until used. The viral titers were 6×10^7 and 8×10^8 plaque forming units/ml for the WSN/33 strain and the R404BP strain, respectively.

Hippocampal cultures

Animals were handled according to Karolinska Institutet's guidelines and experiments were performed with permission from Stockholms "Norra djurförsöksetiska nämnd" (local ethical committee).

Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden), kept under standard laboratory conditions, were sacrificed on the 18th gestational day using carbon dioxide, and the hippocampal regions were dissected from the fetuses. Cultures were then prepared as described previously (Owe-Larsson *et al,* 1999). Briefly, the dissected materials were incubated at 37°C for 15 min in 0.1% trypsin (Gibco, Paisley, UK), diluted in Ca^{2+}-Mg^{2+}–free Hank's balanced salt solution (HBSS, pH 7.3; Gibco) and subsequently triturated through a narrowed Pasteur pipette. Cell suspensions were then seeded into 35 × 10-mm tissue culture dishes (Corning, New York, NY, USA) at a cell density of $3–4 \times 10^5$ cells per dish. Prior to seeding, the dishes were coated with 0.1 mg/ml poly-L-lysine hydrobromide (molecular weight $3–7 \times 10^4$; Sigma) and then washed twice in distilled water.

The cells were cultured in 2 ml of DMEM/Ham's F12, 50:50, (Gibco) supplemented with the following additives: 1.2 mg/ml glucose (BDH, Poole, UK), 10% fetal calf serum (FCS), 5 μg/ml bovine insulin, 100 μg/ml human transferrin, 20 units-μg/ml penicillin/streptomycin (all from Gibco), 20 nM progesterone, 100 μM putrescine, and 30 nM selenium dioxide (all from Sigma). The cultures were maintained in an incubator providing 5% CO_2 at 37°C.

Cell lines
MDCK cells were split weekly after growth in DMEM containing 2 mM L-glutamine, 10% FCS, streptomycin (50 mg/ml), and penicillin (50 IU/ml) (all from Gibco). Neuroblastoma N1E-115 cells (purchased from ATCC, Virginia, USA) were grown in DMEM without sodium pyruvate and containing 10% FCS and 4 mM L-glutamine supplemented with streptomycin and penicillin and incubated in 5% CO_2 at 37°C. The cells were split after 4 to 5 days in culture.

Cloning the NP and NS1 genes and construction of recombinant vectors
NP was cloned from the WSN/33 strain to a cDNA (pCDNA-NP), and NS1 was cloned from the hybrid influenza A virus R404BP strain (pCDNA-NS1). The pCDNA-NP and pCDNA-NS1 were sequenced for both strands, but no substitutions were detected as compared to the published WSN/33 NP (Li *et al*, 1989) and the WSN/33 NS1 sequences (Gene Bank accession number M12597), respectively.

The NP and NS1 genes were then cloned to the expression vector PQE-30 (Qiagen, Hilden, Germany). The sequence for the PTD (33 nucleotides) with four glycines in one recombinant vector was added between the His-tag and the NP or NS1 genes. The GFP gene was PCR-amplified from the p EGFP vector (CloneTech, Palo Alto, CA) and cloned also to the PQE-30 vector. The following primers were used for the PCR amplification (Invitrogen, Leiden, The Netherlands):

1. 5′-gcggtacctatggcaggaagaagcggagacagcgacgaagagatc caaacactg-3′
2. 5′-gcggtaccggaggggaggatatggcaggaagaagc-3′
3. 5′-gcgtcgactcaataccgtccttcttcgcctctgtcgctgcttctaactt ctgacct-3′
4. 5′-gcgtcgacaacttctgacctaattgttcccgcc-3′
5. 5′-gcggtacctcaaacttctgacctaattgttcccgcc-3′
6. 5′-tatggcaggaagaagcggagacagcgacgaagaaagtccctaaga gga-3′
7. 5′-gcggatccgggggaggaggatatggcaggaag-3′

Protein induction and purification
The recombinant vectors were transfected into DH5α *Escherichia coli*. NP-PTD, NS1-PTD, and GFP-PTD proteins were then produced by exposing transfected bacteria to IPTG (isopropyl-β-D-thiogalactopyranoside) (Boehringer Mannheim, Germany) at a final concentration of 0.5 to 1 mM for 6 to 8 h at 37°C. The bacteria were lysed by sonication in a buffer containing (in M) urea, 8; $NaH_2PO_4 \times 2H_2O$, 0.1; Tris, 0.01; imidizole, 0.01; at pH 8.0. The bacterial lysates were centrifuged (12,000 rpm, 4°C, 30 min) and the supernatants added to a His-tag nickel affinity column (Pharmacia Amersham). The proteins were eluted with a buffer containing (in M) urea, 1; $NaH_2PO_4 \times 2H_2O$, 0.1; Tris, 0.01; imidizole, 0.3; at pH 6.3. The eluted proteins were desalted on PD-10 columns (Pharmacia Amersham) in phosphate-buffered saline (PBS) containing 5% glycerol. Protein solutions were membrane-filtered (0.45 μm) (Corning) and protein concentrations were estimated by the Bradford method (BioRad, Hercules, CA, USA). Proteins were verified by Western blotting using anti-NS1 rabbit antiserum (1:1000; a gift from Dr A Nieto, Centro Nacional de Biotecnologia, Madrid, Spain) and anti-NP goat serum (1:100 Virostat, Portland, ME, USA) as primary antibodies, and horseradish peroxidase (HRP) anti-rabbit and HRP anti-goat antisera (both 1:5000; Dako, Glostrup, Denmark) as secondary antibodies. A enhanced chemiluminescent (ECL) kit (Amersham, USA) was used for detection. Aliquots were stored at −70°C.

Exposure of the cultures to the fusion proteins
To allow entry of fusion proteins into N1E-115 cells and hippocampal neurons, the cultures were exposed to the proteins diluted in 600 μl DMEM for 4 h, washed, and further incubated in conditioned cell culture media. For long-term exposure to hippocampal neurons, the fusion protein was added for 4 h every second day. For evaluating toxic effects, the number of neurons in marked squares in the culture dishes was counted daily for 7 days in a phase contrast microscope. The number of counted neurons in each dish varied between 100 and 200.

Immunofluorescence
Cells were fixed in 4% paraformaldehyde in PBS for 15 min. After washing with PBS, they were treated with 0.2% Triton X-100 (Sigma) for 10 min at room temperature (RT), followed by blocking with 5% BSA for 15 min. The primary antibodies used were anti-NS1 rabbit antiserum (1:600; a generous gift from Dr. A. Nieto) and anti-NP goat antiserum (1:200; Virostat). The cells were incubated with the primary antibodies and 1% BSA at 4°C overnight. They were then washed and incubated with the secondary Alexia 488 anti-rabbit antibody (1:200; Molecular Probes, Eugene, OR, USA) or C2 anti-goat antibody (1:200; The Jackson Laboratory, Bar Harbor, Maine, USA) in 1% BSA for 30 to 45 min at RT. The cultures were washed, and samples mounted for observation in a fluorescence microscope. All images were prepared for illustration using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

Electrophysiology
Whole-cell patch clamp recordings were made at RT on neurons selected in an inverted phase–contrast microscope (Nikon TE200, Tokyo, Japan). Neurons having a pyramidal shape with conspicuous processes and a soma width of 15 to 25 μm were chosen for the recordings. The supplemented DMEM culture medium was replaced with an extracellular solution containing (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1; sucrose, 23; and HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), 10 (all

from Sigma). The solution was brought to pH 7.4 with NaOH and osmolarity adjusted to 310 ± 1 mOsm with sucrose.

Voltage clamp records were obtained using an Axopatch 200B patch clamp amplifier (Axon Instruments, Union City, CA, USA). Patch pipettes were pulled to a resistance of 3 to 7 MΩ and filled with an intracellular solution containing (in mM): Potassium gluconate, 140; NaCl, 4; $CaCl_2$, 0.5; $MgCl_2$, 1; EGTA (ethylene glycol-*O*,*O*′-bis-[2-aminoethyl]-*N*,*N*,*N*′,*N*′,-tetraacetic acid), 5; and HEPES, 10; buffered to pH 7.4 with KOH. Osmolarity was adjusted to 305 ± 1 mOsm with sucrose. Gigaseals were made between the recording pipette and the cell membrane by applying slight suction and negative voltage after contact with the cell. Whole-cell configuration was achieved by rupturing the membrane with negative pressure or voltage transients (Hamill *et al*, 1981). The holding potential was −60 mV for sEPSC and mEPSCs, and −40 mV for spontaneous inhibitory postsynaptic current recordings (sIPSC). Series resistance was compensated by at least 80%. The summed access and membrane input resistance was between 200 and 500 MΩ. Command voltages, stimulus current pulses, and digitized data sampling were controlled by an A/D, D/A, and timing computer interface (Digidata 1320A, Axon Instruments) in conjunction with pClamp software (Axon Instruments).

The recordings of sEPSCs and sIPSCs were done in cultures cultivated at three different occasions and at two different occasions for the mEPSCs recordings. From each cultivation occasion, multiple numbers of cultures dishes were used and one to four cells in each of these dishes were recorded from. sEPSCs, sIPSCs, and mEPSCs recordings (120 s of each) were analyzed with the Mini Analysis Program v5.6.22 (Synaptosoft, Decatur, GA, USA). The threshold value for events was set to twice that of the noise level.

Statistical analyses

Statistical analyses were done using two-tailed Student's unpaired *t* test, and one-way analysis of variance (ANOVA) with Bonferronie's *post hoc* test for selected pairs, as appropriate (Graphpad Prism 3.0, Graphpad Software, San Diego, USA).

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