

Pseudorabies virus induces a rapid up-regulation of nitric oxide synthases in the nervous system of swine

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1	PSEUDORABIES VIRUS INDUCES A RAPID UP-REGULATION OF NITRIC OXIDE
2	SYNTHASES IN THE NERVOUS SYSTEM OF SWINE
3	
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1 ABSTRACT

2 Nitric oxide (NO) is a free radical gas with important roles in the host's immune response against 3 viral infections. In this study, we examined the kinetics and distribution of nitric oxide synthase 4 (NOS) expression during the early steps of infection of the porcine nervous system by the 5 alphaherpesvirus pseudorabies virus (PRV). To this end, we examined changes in the expression 6 of the three major NOS isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible 7 NOS (iNOS), by immunohistochemistry in the trigeminal ganglia and brain of pigs inoculated 8 intranasally with a virulent PRV strain. The results obtained show that infection of the porcine 9 nervous system by PRV induced a rapid and progressive increment in NOS expression that 10 coincided in timing, location, and magnitude with those of virus propagation in the nervous 11 tissue. A major finding of this study was that PRV caused not only nNOS and iNOS induction in 12 a variety of cell types, but also eNOS up-regulation in endothelial cells and neurons; therefore, all 13 possible sources of NO are activated and probably contribute to the overproduction of NO during infection with the neurotropic alphaherpesvirus PRV in its natural host. 14

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Keywords: Aujeszky's disease virus; immune response; nitric oxide synthase; nervous system;
trigeminal ganglia; immunohistochemistry.

1 INTRODUCTION

2 A crucial feature shared by all members of the Alphaherpesvirinae subfamily is their 3 ability to invade, propagate, and persist in a latent state in the nervous system of their hosts. It is 4 well known that after primary replication in epithelial cells of mucosal surfaces, the 5 alphaherpesviruses gain access to the termini of nerve fibers innervating the infected tissue, and through axonal transport invade the peripheral ganglia and often the central nervous system 6 7 (CNS), usually resulting in lethal encephalitis. If the host immune response is able to restrain the 8 primary infection, productive infection ceases but the alphaherpesviruses establish a latent 9 infection for the lifetime of their hosts. When the latent infection reactivates, it appears that 10 depending on the immune surveillance at the site of latency, infectious virus can be produced and 11 transported to peripheral tissues (Enquist et al., 1999; Jones, 2003). Therefore, it is clear that the 12 immune system has a key function in the interaction between alphaherpesviruses and their hosts 13 in all the steps of the infectious process, that is during productive and latent infections, as well as in the transitional periods of establishment of and reactivation from latency. 14

15 The defensive molecules deployed by the host immune system during an alphaherpesvirus 16 acute infection have been most thoroughly studied for the prototypical human alphaherpesvirus 17 herpes simplex virus type 1 (HSV-1) using a mouse model of corneal infection. From these 18 studies it was established that, from the mouse cornea, the virus is transmitted to the trigeminal 19 ganglion (TG) within 2 days, reaching peak titers by 3-5 days, and then diminishing to 20 undetectable levels by 7-10 days after infection. HSV-1 replication in the TG is associated with a 21 marked infiltration of macrophages and T lymphocytes and the expression of various 22 immunologically active molecules by these cells, such as the cytokines tumor necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ), and the enzyme inducible nitric oxide synthase 23

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1 (iNOS) (Cantin et al., 1995; Kodukula et al., 1999; Liu et al., 1996; Shimeld et al., 1995 and 1997). Evidence that the innate immunity controls primary virus replication in the mouse TG was 2 3 provided by neutralization of TNF- α or IFN- γ or inhibition of NO production by iNOS, resulting in a dramatic increase in HSV-1 titers in the TG (Kodukula et al., 1999). Although both TNF- α 4 5 and IFN- γ show direct antiviral activity against HSV-1, their main antiviral effect is to stimulate iNOS up-regulation in immune cells, and therefore, to induce the production of NO at sites of 6 7 virus infection (Ellermann-Eriksen, 2005; Feduchi and Carrasco, 1991; Feduchi et al., 1989; 8 Karupiah et al., 1993).

The role played by NO in the pathogenesis of virus infections has been, however, 9 questioned during the past years, since both protective and cytotoxic effects of this molecule are 10 11 frequently seen in parallel (Bogdan, 2001; Guix et al., 2005; Thippeswamy et al., 2006). The 12 antiviral effect of NO seems to be provided by its ability to inhibit virus replication through 13 suppression of viral mRNA and protein synthesis, probably by inhibition of the enzyme 14 ribonucleotide reductase (Croen, 1993; Komatsu et al., 1999b). In line with this, it was shown 15 that mice deficient in iNOS or treated with a NOS inhibitor were more susceptible to HSV-1 16 infection compared with similarly infected control animals (Adler et al., 1997; Gamba et al., 17 2004; MacLean et al., 1998). On the other hand, an increasing body of evidence indicates that 18 NO is toxic, since it allows generation of a highly reactive nitrogen oxide species, peroxynitrite 19 $(ONOO^{-})$, by reaction of NO with superoxide (O_2^{-}) . Peroxynitrite causes cytotoxic effects and 20 tissue damage through potent oxidation and nitration of various biomolecules, thus contributing 21 to pathogenic processes (Akaike and Maeda, 2000; Zaki et al., 2005). This cytotoxic effect of NO 22 has been pointed out following HSV-1 infection of the brain (Fujii et al., 1999; Meyding-Lamadé 23 et al., 2002), and also in HSV-1-induced pneumonia (Adler et al., 1997).

1 In recent years, it has been demonstrated that not only iNOS, but also the other two major 2 NOS subtypes neuronal NOS (nNOS) and endothelial NOS (eNOS) operate as players of the 3 immune system (Bogdan, 2001). The three isoforms synthesize NO, but they mainly differ with 4 respect to their regulation, amplitude and duration of the production of NO: nNOS and eNOS are Ca2+-calmodulin-dependent and generate low amounts of NO lasting a few minutes, whereas 5 iNOS is controlled by inflammatory mediators and cytokines and produces large quantities of NO 6 7 for hours or days, thus being responsible for the overproduction of NO during an immune 8 response (Guix et al., 2005). In the case of neurotropic viral infections, in addition to immune 9 cells that infiltrate the nervous tissue and generate large quantities of iNOS-derived NO, it has 10 been observed an increment in the number of neurons that express nNOS in virus-infected areas 11 (Komatsu et al., 1999a; Serrano et al., 2002); the up-regulation of eNOS has also been reported in 12 neurons and astrocytes, cell types that usually do not express this isoform in the uninfected 13 nervous tissue (Barna et al., 1996; Shin et al., 2004). In this context, the present study set out to 14 investigate the dynamics and sources of NO production during the initial stages of an 15 alphaherpesvirus invasion of the nervous system, with the aim to achieve a better understanding 16 of the possible role(s) played by NO in the pathogenesis of the infection. To this end, we used a 17 virulent strain of the alphaherpesvirus pseudorabies virus (PRV) (Suid herpesvirus 1) and 18 infected its natural host, swine. Our results show that PRV infection of the porcine nervous 19 system induced a rapid and progressive up-regulation of NOS in a variety of cell types, and that 20 the three NOS isoforms may contribute to the overproduction of NO in the infected nervous 21 tissue.

1 MATERIALS AND METHODS

Animals and virus. The study was conducted using seven 2-month-old conventional pigs that 2 3 were not vaccinated against PRV and were determined to be seronegative to PRV by enzymelinked immunosorbent assay. They were caged individually under strict isolation containment, 4 5 with water and commercial food freely available. Six pigs were inoculated intranasally with the high-virulence field strain of PRV E-974 isolated in northwestern Spain from nervous tissue of 6 7 naturally infected pigs. Information about the use of this strain has been published previously 8 (Alemañ et al., 2001; Marcaccini et al., 2006). The titer of the virus suspension, determined on BHK-21 cells, was 10^{6.5} 50% tissue culture infective doses of PRV-E-974 per ml. Each 9 10 inoculated animal was given 2 ml of virus suspension, 1 ml in each nostril. The pigs were 11 euthanized with an intravenous injection of sodium pentobarbital at 24, 48, and 72 h 12 postinoculation (hpi), two animals at each time point. The remaining uninfected pig was killed at 13 the beginning of the experiment and served as a negative control. At necropsy, the TG and brain were removed, fixed by immersion in 10% buffered formalin for 24 h, and then coronal slices 14 15 were embedded in paraffin according to standard laboratory procedures.

Light microscopy. For histological examinations, paraffin-embedded nervous tissue samples
were sectioned at 4 µm and stained with hematoxylin and eosin.

Immunohistochemistry. Tissue sections placed on silanized slides were deparaffinized with xylene, rehydrated through a graded alcohol series, and incubated with H_2O_2 (DakoCytomation, Denmark) for 30 min at RT to inactivate endogenous peroxidase activity. The antibodies used to identify on paraffin sections PRV-infected cells, the three NOS isoforms (nNOS, eNOS, iNOS), astrocytes (glial fibrillary acidic protein, GFAP), T lymphocytes (CD3) and macrophages (MAC387) are shown in Table 1. All antibodies were incubated overnight at 4°C. Antigen-

antibody complexes were detected with a peroxidase-conjugated polymer which carries 1 2 secondary antibody molecules directed against rabbit or mouse immunoglobulins (EnVisionTM+, 3 DakoCytomation) applied for 30 min at RT, and color development was achieved by incubating 4 sections with diaminobenzidine (DAB, DakoCytomation). Finally, the slides were counterstained 5 with hematoxylin, dehydrated, and permanently mounted in DePeX (BDH Laboratory Supplies, 6 Poole, United Kingdom). We also performed double immunostaining for PRV, combined with 7 nNOS, eNOS or iNOS. This technique closely followed the staining protocol described above, using DAB (brown) to visualize the PRV antibody, and Vector SG (blue) (Vector Laboratories, 8 9 Burlingame, CA) to develope the NOS antibodies. Double-immunolabeled slides were 10 counterstained with 1% methyl green to visualize the overall cytoarchitecture. In each series of 11 immunostained slides, positive- and negative-control sections were included to assess the 12 specificity of the assay. As positive controls, sections of nervous tissue from pigs infected with 13 strain E-974 in a previous experiment were used for PRV detection; for staining of the NOS isoforms, astrocytes, T cells, and macrophages, sections of human tonsil, porcine nervous and 14 lymphoid tissues (from the uninfected pig and from other healthy animals not related with this 15 16 experiment), and rat nervous tissue were used. As negative controls, primary antibodies were 17 replaced by buffer or, for the anti-PRV antibody, sections from the uninfected animal were used.

1 **RESULTS**

2 **PRV** propagation in nervous tissue and production of encephalitis

3 Histological changes in the TG and brain of the inoculated pigs consisted of a nonsuppurative 4 ganglioneuritis and encephalitis whose magnitude correlated directly with the advancing survival 5 time of the animals and the extent of the infection. As expected, the topographical distribution of the lesions and virus antigens was dependent on the route PRV took to the CNS from the nasal 6 7 cavity, i.e., they were found in structures related with the trigeminal and main olfactory nervous 8 pathways: TG, spinal nucleus and tract of the trigeminal nerve in the brain stem, and olfactory 9 bulb (Figure 1A, B and C). In the TG, it became apparent a progressive infiltration of 10 mononuclear immune cells, mainly T lymphocytes and macrophages, that caused confluent foci 11 of inflammation containing neurons at different stages of neuronophagia. In the brain, lesions 12 also increased in a time-dependent manner and consisted of perivascular aggregations of 13 increasing numbers of T cells and macrophages (Figure 1D and E), and both diffuse and focal gliosis (Figure 1F), but we only observed a few images of satellitosis and neuronophagia at 72 14 hpi. During these early phases of virus replication, PRV-infected neurons were scarce, except in 15 16 the TG where a large number of infected ganglionic neurons were seen at 48 and especially at 72 17 hpi.

The E-974 strain of PRV propagated more efficiently through the trigeminal than through the olfactory nervous pathway, as revealed by the differential appearance of lesions and virus antigens in each nervous circuitry. At 24 hpi, lesions and PRV-infected cells were only detected in the TG. In this organ, the magnitude of the inflammatory reaction and the extent of PRV infection greatly increased at 48 and 72 hpi (Figure 1A). In the brain stem, lesions and infected cells were first observed at 48 hpi in the pontine root and spinal tract of the trigeminal nerve; at 72 hpi, the infection involved more rostral and caudal segments in the brain stem, and also

affected the pontine and spinal nucleus of the trigeminal nerve (Figure 1B) and the medial lemniscus. On the other hand, in the olfactory bulb, perivascular cuffs and focal gliosis were first detected in the nerve and glomerular layers at 48 hpi, lesions that at 72 hpi were also present in deeper layers. In this structure, PRV-infected cells were scarce and they were mainly identified as infiltrating immune cells and glial cells (Figure 1C), with only a few infected periglomerular neurons at 48 hpi, and very few infected mitral cells at 72 hpi.

7 NOS expression in uninfected nervous tissue

8 The three antibodies against the NOS isoforms used in this study reacted with nervous structures 9 in the TG and brain tissue of the uninfected porcine nervous tissue. While eNOS expression was 10 restricted to the endothelial lining of the majority of small and medium size vessels (Figure 2A), 11 nNOS expression was detected in the perikaryon of some TG neurons, in a moderate number of 12 small neurons located in various nuclei of the brain stem, and in a few periglomerular neurons of 13 the olfactory bulb (Figure 2B and C). In the case of the iNOS isoform, the antibody recognized the muscular layer of most medium size blood vessels, a few neurons in the TG (Figure 2D), and 14 15 scattered neurons and astrocytes in the brain stem and in the granule cell layer of the olfactory 16 bulb. Immunohistochemical staining of serial sections from TG and brain stem revealed that the 17 majority of nNOS and iNOS expressing neurons were different populations (Figure 2C and D), 18 although we also observed a few neurons that stained for both isoforms (data not shown).

19 eNOS expression in PRV-infected nervous tissue

There were no changes in eNOS expression at 24 hpi relative to that observed in the uninfected nervous tissue. At 48 hpi, increased expression of eNOS became evident in TG and brain tissue both as an increment in the number of immunoreactive blood vessels and as a stronger intensity of staining (Figure 3A and B). Notably, eNOS expression in endothelial cells was of greater magnitude in those vessels of the brain stem and olfactory bulb that showed perivascular cuffs of

mononuclear immune cells (Figure 3B). At this time postinoculation, expression of eNOS was also detected in scattered neurons situated in areas of PRV-induced damage in the brain stem (Figure 3B) and in the mitral cell layer of the olfactory bulb. The number of eNOS immunopositive neurons was increased in the same areas at 72 hpi, but we did not observe simultaneous eNOS expression in the low number of neurons that stained for PRV. Although PRV-infected neurons were numerous in the TG, especially at 72 hpi, ganglionic neurons did not exhibit eNOS immunoreactivity at any time point investigated.

8 nNOS expression in PRV-infected nervous tissue

9 Expression of nNOS in the nervous tissue of PRV-infected pigs was already altered at 24 hpi. 10 Compared with control sections, there was a larger number of nNOS-immunopositive neurons in 11 the TG, in various nuclei of the brain stem and, in the olfactory bulb, in periglomerular neurons 12 and in neurons of the granule cell layer. At 48 and 72 hpi, nNOS expression remained elevated in 13 the TG (Figure 3C) and olfactory bulb, whereas in the brain stem the number of nNOS immunopositive neurons and the intensity of staining increased further to high levels in both 14 15 areas of PRV-induced damage and adjacent noninjured areas. In the TG, we could identify 16 morphologically healthy neurons that only stained for nNOS, as well as neurons that stained both 17 for nNOS and PRV and exhibited morphological changes to a greater or lesser extent (Figure 3C 18 and D); however, we did not observe nNOS expression in neurons during the process of neuronophagia (Figure 3C and D). 19

20 iNOS expression in PRV-infected nervous tissue

In the TG, an increase in iNOS expression was already detected at 24 hpi. At this time postinoculation, iNOS immunoreactivity was observed in a higher number of TG neurons in comparison with that seen in the control sections and, in addition, a few infiltrating immune cells also expressed iNOS. Expression of iNOS in the TG markedly increased until 72 hpi mainly as a

1 result of the increment in the number of immunopositive infiltrating immune cells, but a few 2 satellite cells located within foci of inflammation also displayed iNOS immunoreactivity (Figure 3 4A). In the same way as for nNOS expression, we identified PRV-infected neurons 4 simultaneously immunolabeled for iNOS, but we could not detect neuronal iNOS expression in 5 foci of advanced neuronophagia (Figure 4A and B). In both the brain stem and olfactory bulb, the 6 number of iNOS immunopositive neurons and astrocytes was increased at 24 hpi. A significant 7 increase in iNOS expression by neurons and astrocytes became apparent at 48 hpi especially in 8 areas of PRV-induced pathology, but also in adjacent nondamaged areas (Figure 4C). At this time 9 postinoculation, mononuclear immune cells located in perivascular cuffs and in foci of gliosis 10 (Figure 4D), as well as cells with characteristic microglial cell morphology located at sites of 11 PRV infection also exhibited iNOS immunoreactivity. At 72 hpi, the number of iNOS 12 immunopositive neurons, astrocytes, microglial cells and immune cells was substantially larger, showing a distribution that correlated differentially with the sites of PRV-induced pathology. 13

14

1 DISCUSSION

We have studied the initial phases of PRV infection in its natural host, stages in which the 2 3 alfaherpesvirus invades the nervous system and the host's innate defense mechanisms play a 4 crucial role in determining future events in the antiviral immune response and the final outcome 5 of infection. The results obtained herein show that PRV infection of the porcine nervous system induced a rapid up-regulation of the three major NOS isoforms, both by nervous tissue cells 6 7 situated in and by immune cells recruited into virally infected structures of the nervous system. In 8 addition, the timing, location, and magnitude of NOS expression coincided with the progression 9 of PRV infection and, consequently, the number of cells that can produce NO in the nervous 10 system increased in a time-dependent manner. A major finding of this study is that PRV infection 11 not only induced the up-regulation of iNOS and nNOS, but also an increment in the expression of 12 the eNOS isoform.

Regarding eNOS and nNOS, PRV infection of the porcine nervous system resulted in an 13 enhanced expression of these isoforms in nervous tissue cells that expressed low levels or, 14 15 alternatively, did not express these isoforms in the uninfected nervous tissue. In the case of 16 eNOS, a marked increase in the number of immunoreactive vessels and in the intensity of 17 staining was evident in infected animals at 48 hpi, indicating that eNOS expression is up-18 regulated in endothelial cells during the first steps of acute PRV infection. A similar up-19 regulation of eNOS in the nervous system has been documented in mice infected with vesicular 20 stomatitis virus (Komatsu et al., 1999a), and during the course of bacterial meningitis (Winkler et al., 2001). In a study performed by Koedel and colleagues (2001), it was demonstrated that eNOS 21 22 expression has a protective effect on the course of pneumococcal meningitis, since infected 23 eNOS-null mice showed a more pronounced leukocyte infiltration into the cerebrospinal fluid 24 and blood-brain barrier disruption than infected wild type mice. This finding, together with the

1 fact that in a subsequent study they found that bacterial meningitis causes an increase in eNOS mRNA and protein (Winkler et al., 2001), prompted these authors to suggest that eNOS up-2 3 regulation may contribute to counteract the leukocyte infiltration and blood-brain barrier 4 breaching observed in this disease. This interpretation is consistent with one of the roles ascribed 5 to eNOS-derived NO in physiological conditions, i.e., to regulate the interaction between the endothelium and immune cells through inhibiting leukocyte adhesion and infiltration (Arnal et 6 7 al., 1999; Cirino et al., 1993; Moro et al., 2004). In this respect, it is worth mentioning that we 8 observed the highest level of eNOS expression in blood vessels surrounded by perivascular cuffs 9 of mononuclear cells and located in PRV-infected areas.

10 An unexpected finding of this study was to observe brain neurons exhibiting eNOS 11 immunoreactivity located in areas of virus infection and injury. These eNOS immunopositive 12 neurons were first detected at 48 hpi and their number increased at 72 hpi. The occurrence of 13 neurons that express eNOS in physiological conditions has been reported previously in the rat olfactory bulb, cerebellum and hippocampus (Dinerman et al., 1994; Doyle and Slater, 1997). 14 However, we did not observe eNOS immunoreactive neurons in the uninfected porcine brain or 15 16 in the infected animals at 24 hpi, and therefore, our results clearly indicate that PRV infection 17 caused eNOS up-regulation in brain neurons that did not express this NOS isoform in the 18 uninfected nervous tissue. A similar up-regulation of eNOS expression in neurons has been 19 documented in the brain of cattle infected with rabies virus (Shin et al., 2004), and in other 20 nervous cell types, such as astrocytes and ependymal cells in mice after infection with vesicular 21 stomatitis virus (Barna et al., 1996; Komatsu et al., 1999a).

Regarding nNOS, PRV infection induced an increment in its expression that took place earlier than eNOS up-regulation, since the number of neurons that expressed this isoform was increased at 24 hpi in TG, brain stem, and olfactory bulb. We found that the number of nNOS

1 immunoreactive neurons increased further only in the brain stem at 48 and 72 hpi. Indeed, the brain stem was the structure of the CNS that contained the greatest number of PRV-infected cells 2 3 and showed lesions of higher magnitude at the last time postinoculation investigated, a finding 4 that points to a more efficient virus spread of the strain used in this study through the trigeminal 5 than through the olfactory nervous pathway following an intranasal inoculation. Therefore, the magnitude and spatiotemporal evolution of nNOS expression correlated with those of PRV 6 7 propagation in the CNS. The up-regulation of nNOS expression in neurons has been documented 8 in neurotropic viral and bacterial infections (Komatsu et al., 1999a; Serrano et al., 2002; Shin et 9 al., 2000). In the study performed by Serrano and colleagues (2002) in rats infected with PRV, 10 they found that the induction of nNOS occurred exclusively in infected neurons of the brain stem. 11 In our study, however, we observed that nNOS immunoreactive neurons exceeded in number that 12 of infected neurons in all structures and at any time point investigated. We can not explain the 13 different pattern of nNOS expression between PRV-infected rats and swine, but it has to be considered that it may be a consequence of the various PRV strains used in both studies and, 14 15 more important, that the pig is the natural host of the virus and the only species capable of 16 surviving a primary infection (Pensaert and Kluge, 1989; Wittmann and Rziha, 1989). In this 17 regard, it is becoming evident that the increase in nNOS-mediated NO production may represent 18 a neuroprotective mechanism, and may be considered as essential for cell survival and recovery 19 (Keilhoff et al., 2004; Kim et al., 2000). For example, it has been shown that nNOS expression in 20 neurons is associated with survival of mice from vesicular stomatitis virus infection (Komatsu et 21 al., 1999a). Also, in a recent study, it was demonstrated that deficiency of nNOS leads to a 22 substantial apoptotic cell death of spinal cord neurons after peripheral axotomy (Keilhoff et al., 23 2004); among the plausible explanations for this phenomenon, Keilhoff and colleagues (2004) 24 argued that the lack of NO supply causes a loss of the inhibitory effect of NO at the level of the

1 initiator and/or effector caspases of the cell death program. In line with this, we have previously shown that the active form of the effector caspase-3 is only expressed in PRV-infected neurons at 2 3 advanced stages of neuronophagia (Marcaccini et al., 2006), and it is worth noting that precisely 4 during advanced neuronophagia TG neurons did not exhibit immunoreactivity for any NOS 5 isoform in the present study. As viral mRNA and protein synthesis is impaired by NO (Croen, 1993; Komatsu et al., 1999b), it is tempting to speculate that PRV-infected neurons that do not 6 7 synthesize endogenous NO dye by apoptosis at late stages of the replication cycle, whereas those 8 that synthesize NO may be more resistant to the infection or, alternatively, survive the infection 9 and harbor the virus in the latent state. Therefore, NO might be a factor that contributes to the 10 survival of infected neurons and to the establishment and maintenance of alphaherpesvirus 11 latency. This contention is in line with the results reported by Gao and colleagues (1999), who 12 demonstrated that endogenously synthesized NO helps to maintain Epstein-Barr virus latency in 13 epithelial cell lines.

The majority of the cells that expressed iNOS were identified among immune cells; they 14 were most probably activated macrophages (Guix et al., 2005; Thippeswamy et al., 2006) 15 16 recruited to sites of virus infection and located in perivascular cuffs and within foci of 17 inflammation. They were first found at 24 hpi in TG, and at 48 hpi in the brain stem and olfactory 18 bulb, and since the magnitude of the inflammatory reaction increased in a time-dependent 19 manner, iNOS-expressing cells were present in higher numbers in areas of PRV-induced damage 20 as the time postinoculation increased. Besides immune cells, it was apparent the existence of 21 additional sources of iNOS-derived NO. In the healthy porcine nervous system, a few neurons 22 and astrocytes exhibited iNOS immunoreactivity in TG, brain stem and olfactory bulb; a 23 moderate increment in its number was noticed as early as at 24 hpi in all three structures, but it 24 was at higher postinoculation times when they were significantly increased in infected pigs

specially at sites of virus-induced injury. Also, satellite cells located within foci of inflammation in the TG, and cells exhibiting microglial cell features in foci of gliosis were found to express iNOS. Again, the kinetics and distribution of iNOS expression in immune cells and in cells of the nervous parenchyma were in agreement with those of PRV spread in the porcine nervous system.

5 Induction of iNOS up-regulation in infiltrating macrophages has already been reported after infection of the brain with the alphaherpesviruses PRV (Serrano et al., 2002) and HSV-1 6 7 (Fujii et al., 1999), as well as with other neurotropic viruses (Akaike et al., 1995; Campbell et al., 8 1994; Gómez et al., 2003; Komatsu et al., 1999a; Koprowsky et al., 1993; Zheng et al., 1993). 9 Similarly, the expression of iNOS in glial cells has also been described under both normal and 10 various pathological circumstances, including during the course of viral infections (Fujii et al., 11 1999; Gómez et al., 2003; Goody et al., 2005; Komatsu et al., 1999a; Lin et al., 1997; Serrano et 12 al., 2002). To our knowledge, however, the expression of iNOS in healthy neurons has only 13 exceptionally reported in rat (Chen et al., 2003). In this study, immunohistochemical staining of serial sections from the uninfected porcine nervous tissue resulted in the identification of neurons 14 that expressed either nNOS or iNOS, a few neurons that expressed both isoforms, and neurons 15 16 that did not express either NOS isoform. As hypothesized above, the expression of a NOS 17 isoform and, therefore, the synthesis of endogenous NO, might represent a difference in the 18 infectious events occurring in a particular neuron. But more importantly, our approach 19 demonstrated that PRV infection of the porcine nervous system induced a rapid increment in the 20 number of neurons that expressed the iNOS isoform. The expression of iNOS by neurons has been demonstrated in the injured rodent brain both in vitro and in vivo (Minc-Golomb et al., 1994 21 22 and 1996; Moro et al. 1998), and to date there are two additional reports of neurons expressing 23 iNOS during rabies virus (Shin et al. 2004) and reovirus infection (Goody et al., 2005). This 24 iNOS up-regulation in macrophages, and likewise in nervous tissue cells, is thought to be

1 mediated by proinflammatory cytokines, such as IFN- γ , TNF- α and interleukins, as well as 2 directly induced by the virus (Akaike and Maeda, 2000; Zaki et al., 2005). It is of interest to 3 emphasize that the number of iNOS expressing cells increased in a time-dependent manner, and consequently at 72 hpi we observed large numbers of different cell types expressing iNOS. Since 4 the iNOS isoenzyme produces high amounts of NO (10-100 times more) and for a longer period 5 6 of time than do nNOS and eNOS, and the activity of NO is strongly influenced by its 7 concentration (Akaike and Maeda, 2000; Bogdan, 2001; Guix et al., 2005), it is clear that the 8 initial antiviral effect of NO would be progressively obscured by its pathological effects via its 9 interaction with reactive oxygen species, producing nitrogen oxides and therefore nonspecific 10 oxidative and nitrative injury (Akaike and Maeda, 2000; Zaki et al., 2005).

11 In summary, we have established that the alphaherpesvirus PRV induces a rapid and 12 progressive up-regulation of NOS expression in a variety of cell types during the initial stages of 13 its propagation in the nervous system of swine. PRV causes not only iNOS and nNOS induction, 14 but also eNOS up-regulation, a finding that indicates that all possible sources of NO are activated 15 in the porcine nervous system following infection with the neurotropic alphaherpesvirus PRV. 16 Another interesting result from the present study is the suggestion that the expression of the 17 nNOS and/or iNOS isoforms by neurons, and therefore, the presence of endogenously 18 synthesized NO, might represent a pathogenic factor in the infectious process by contributing to the survival of PRV-infected neurons and/or to the establishment and maintenance of virus 19 20 latency.

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Specificity	pAb/mAb	Treatment	Dilution	Source
PRV	pAb	None	1/1,500	M.B. Pensaert, University of Ghent, Belgium
nNOS	pAb	None	1/200	Serotec, Oxford, UK
eNOS	pAb	Heat under pressure for 15 min in 0.1 M citrate buffer, pH 6.0	1/50	NeoMarkers, Fremont, CA
iNOS	pAb	Heat under pressure for 15 min in 0.1 M citrate buffer, pH 6.0	1/200	NeoMarkers, Fremont, CA
GFAP	pAb	Proteinase K (DakoCytomation, Denmark) digestion for 5 min at RT	1/5,000	DakoCytomation, Denmark
CD3	pAb	Heat under pressure for 15 min in 0.1 M citrate buffer, pH 6.0	Prediluted	DakoCytomation, Denmark
MAC387	mAb	0.1% trypsin (Sigma, Spain) digestion for 45 min at 37°C	1/200	Serotec, Oxford, UK

2

3 **Table 1.** Details of the antibodies used in the study to identify PRV-infected cells, the three NOS

4 isoforms (nNOS, eNOS, iNOS), astrocytes (GFAP), T lymphocytes (CD3) and macrophages

5 (MAC387). pAb/mAb, polyclonal antibody/monoclonal antibody.

Figure 1. Immunohistochemical detection of PRV antigens in TG (**A**), brain stem (**B**), and olfactory bulb (**C**) at 72 hpi. (**D**, **E** and **F**) Lesions in the brain stem of PRV-infected pigs at 72 hpi showing immunostaing for T lymphocytes (**D**, anti-CD3 antibody), macrophages (**E**, anti-MAC387 antibody), and reactive astrocytes (**F**, anti-GFAP antibody). G, glomerular layer of the main olfactory bulb. Original magnifications, X4 (A and C), X10 (B), and X20 (D, E and F).

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Figure 2. Immunohistochemical detection of eNOS (A), nNOS (B and C) and iNOS (D) in the
TG (A, C and D) and olfactory bulb (B) from healthy porcine nervous system. Note that neurons
immunopositive for nNOS in panel C (arrows) are not stained for iNOS on the consecutive
section showed in panel D (arrows). G, glomerular layer of the main olfactory bulb. Original
magnifications, X4 (B), and X10 (A, C and D).

12

Figure 3. Immunohistochemical detection of eNOS (A and B), and nNOS (C and D) in the 13 nervous tissue of PRV-infected pigs. (A) Section from TG at 48 hpi showing numerous eNOS 14 15 immunopositive blood vessels. (B) Brain stem at 48 hpi. Note the strong intensity of staining for 16 eNOS in endothelial cells of vessels that show perivascular cuffs. The inset shows a brain stem 17 neuron immunopositive for eNOS. (C) nNOS immunoreactivity in TG neurons at 48 hpi. Note 18 that neurons during the process of neuronophagia (arrows) do not exhibit immunoreactivity for 19 nNOS. (D) Double immunostaining for PRV (brown stain) and nNOS (blue stain) in the TG at 72 20 hpi. The arrow points to a double-stained neuron; the arrowhead points to a neuron only stained 21 for PRV and in process of neuronophagia. Original magnifications, X10 (A, B, C, and D), X20 22 (inset in C), and X40 (inset in B).

23

1 Figure 4. Immunohistochemical detection of iNOS in the nervous tissue of PRV-infected pigs. 2 (A) Section from TG at 48 hpi showing numerous iNOS immunopositive neurons, immune cells 3 and an immunoreactive satellite cell (arrow). Note that the neuron within the focus of 4 neuronophagia (arrowhead) does not stain for iNOS. (B) Two ganglionic neurons showing 5 simultaneous detection of PRV antigens (brown stain) and iNOS (blue stain) at 72 hpi. (C) 6 Section from a PRV-noninjured area of the brain stem at 48 hpi showing a nucleus comprised of 7 iNOS immunopositive neurons. (D) iNOS expression in perivascular immune cells and in foci of gliosis in the brain stem at 72 hpi. Original magnifications, X10 (C and D), and X20 (A and B). 8







