The Role of Nectin-1 and Oxidative Damage in the Development of Herpes Simplex Encephalitis

Ph.D. Thesis

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PUBLICATIONS AND PRESENTATIONS

The following publications are included by the thesis:

I. **Prandovszky, E**, Horvath S, Gellert, L, Kovacs SK, Janka, Z, Toldi,J, Shukla,D, Valyi–Nagy, T.
Nectin-1 (HveC) is expressed at high levels in neural subtypes that regulate radial migration of cortical and cerebellar neurons of the developing human and murine brain. *J. Neurovirol.*14: 1–9, 2008. (Impact factor: 3.290)

II. Kavouras, JH, **Prandovszky, E**, Valyi-Nagy, K, Kovacs, SK, Tiwari, V, Kovacs, M, Shukla, D, Valyi-Nagy, T [1]
HSV-1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures
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III. Horvath, S, **Prandovszky, E**, Kis, Z , Krummenacher, C, Eisenberg, RJ, Cohen,GH, Janka, Z, Toldi, J [2]
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V. Horvath, S, **Prandovszky, E**, Pankotai, E, Kis, Z, Farkas,T, Boldogköi,Z, Boda,K, Janka, Z, Toldi, J.
Use of a recombinant pseudorabies virus to analyze motor cortical reorganization after unilateral facial denervation
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[1] Kavouras JH and Prandovszky E equally contributed to this work
[2] Horvath S and Prandovszky E equally contributed to this work

I have performed the following presentations:

<u>**Prandovszky, E**</u>, O'Donnell, C, Horvath, Sz, Janka, Z, Valyi-Nagy, T.
Cell type and differentiation dependent modulation of herpes simplex virus type 1 (HSV-1) replication by lipid peroxidation by-product, 4-hidroxy-2-nonenal
MÉT-FÉKF 2007, Pécs

<u>**Prandovszky, E**</u>, Tiwari,V, Shukla,D, and Valyi–Nagy, T
Expression of Herpesvirus Entry Mediator (HVEM) in the Retina and Trigeminal Ganglia
ARVO 2006, Ft.Lauderdale

Valyi–Nagy,T, Tiwari,V, <u>**Prandovszky,E,**</u> Shukla, D.
Expression of Herpesvirus Entry Mediator (HVEM) in Normal and Herpes Simplex Virus Type 1–Infected Cornea
ARVO 2006, Ft.Lauderdale

<u>**Prandovszky, E,**</u> Horvath, S, Janka, Z, Toldi, J
Poliovirus infection induced schrizophrenia: is nectin-1 the missing link?
MIT 2004, Budapest

I. ABBREVIATIONS

AC   anterior commissure
CA   cornu ammonis
CC   corpus callosum
CH   commissura hippocampi
CHO   Chinese Hamster Ovary
CMS   callosal migratory stream
CNS   central nervous system
CP   cortical plate
CR   Cajal-Retzius cells
CTX   cortex
DG   dentate gyrus
DMEM   Dubelco's Modified Essential Media
FBS   fetal bovine serum
GFP   green fluorescent protein
HNE   4-hydroxy-2-nonenal
HPF   hydroxyphenyl fluorescein
HRP   horseradish peroxidase
HSE   Herpes Simplex Encephalitis
HSV   Herpes Simplex Virus
MDA   malondialdehyde
MZ   marginal zone
OB   olfactory bulb
p.i.   post infection time
P0, P7   postnatal day 0, 7
P19N   neurally differentiated P19 cell line
PBS   phosphate-buffered saline
PFA   paraformaldehyde
RMS   rostral migratory stream,
ROS   reactive oxygen species
RT   room temperature
SP   subplate.
SVZ   subventricular zone
UV   ultraviolet light
VZ   ventricular zone

II. RESEARCH AIMS

Herpes simplex virus type–1 and –2 (HSV-1 and HSV-2) are neurotropic viruses belonging to a subfamily of herpesviruses known as alphaherpesviruses. Though commonly known for causing oral (HSV-1) and genital (HSV-2) lesions, HSV infection can spread to the central nervous system (CNS) and cause life threatening illnesses, including meningitis and herpes simplex encephalitis (HSE). HSE is one of the most common causes of sporadic fatal encephalitis in humans. While HSV-1 is the more common cause of adult encephalitis, HSV-2 is more likely to cause newborn encephalitis. HSV-1 infection induces HSE in adults and children older than three months, which is localized primarily to the temporal and frontal lobes. However, HSV-2 mediated HSE occurs in neonates and brain involvement is generalized. Evidence suggests HSV receptor expression pattern may play a significant role in this difference between HSV-1 and HSV-2 mediated HSE, but that role is currently unknown.

Despite recent advancements in antiviral therapy in the past two decades, HSE remains a serious illness with significant risks of morbidity and death. The mortality rate in untreated patients is about 70%. Among treated patients, the mortality rate is 19%, and more than 50% of survivors are left with moderate or severe neurological defects. About one in three cases of HSE is due to primary herpes simplex virus (HSV) infection. The remaining two-third occurs in patients with serologic evidence of preexisting HSV infection and is due to either reactivation of a latent peripheral infection in the olfactory bulb or trigeminal ganglion, or reactivation of a latent infection in the brain itself.

The pathogenesis of HSE is poorly understood. Previous studies have reported that neurons are quickly overwhelmed by a lytic and hemorrhagic process distributed in an asymmetric fashion throughout the medial temporal and inferior frontal lobes. The exact mechanism of cellular damage is unclear, but it may involve both direct virus-mediated and indirect immune-mediated inflammatory processes. Tissue damage due to oxidative stress can be induced by viral infection. It is possible HSV infection can also cause oxidative tissue damage and this could contribute to HSE. Evidence suggests that viral infection spreads rapidly within limbic structures, probably starting at one side of the brain, and spreading within it to the other side. This process, which lasts about three

weeks, leaves behind a trail of severe necrosis and inflammation in infected parts of the brain. It is believed that certain host and viral factors involved in HSV infection play a significant role in HSE pathogenesis, but, as of now, that role is unknown.

Certain host and viral factors, specifically glycoproteins expressed on the viral envelope, are critical for HSV entry and cell-cell spread. HSV entry is composed of two steps: i) initial attachment to the plasma membrane and ii) subsequent fusion of the viral envelope to either the plasma membrane or endosomal membrane after entry via endocytosis. Viral glycoproteins are critical for these steps, as well as, the receptors for HSV, which includes nectin-1 and nectin-2, HVEM, 3-O-sulfated heparan sulfate. In neuronal cells, the primary receptor for HSV-1 and HSV-2 infection is nectin-1. These receptors interact with HSV glycoprotein D (gD) to mediate entry. RhoA small GTPases, which can be activated by nectin-1, as well as oxidative reactive species (ROS), are involved in signaling pathways that lead to oxidative tissue damage and thus, could be activated by HSV during infection and contribute to HSE.

All together, evidence suggests that the function of viral glycoproteins during HSV-1 entry and spread, as well as, the function and expression pattern of host cell receptors, specifically nectin-1, plays a critical role in the development and pathogenesis of HSE. That exact role, however, is currently unknown. Our study will focus on these viral and host factors, as well as, the molecular mechanisms these factors are involved in, such as oxidative stress and Rho GTPase signaling, to study their roles in HSE. At this point, there is no available medication to heal all of the neurological problems associated with HSE. Therefore, it is critical to identify the cellular and molecular mechanism of entry and cell-cell spread of HSV-1, and these findings may contribute to the development of therapeutic agents to prevent HSE.

The aims and scopes of my work are as follows:

1. **To test the hypothesis that host cell HSV receptor expression pattern is responsible for the difference in pathogenesis seen in newborn and adult type of HSE, we determined the spatiotemporal changes in the distribution of the major neural entry receptor for HSV (nectin-1) in postnatal mouse brains of newborn and adult BALB/c mice**

2. **Compare human and mouse samples to identify similar expression patterns of nectin-1 in both developing CNS (esp. hippocampus and cortex).**

3. **To determine a possible molecular mechanism for HSE development, we examined the impact of HSV-1 infection on oxidative tissue damage and its contribution to the development of HSE.**

4. **To investigate how lipid peroxidation by-products already present in the cell affect HSV-1 infectivity in neuronal and non-neuronal cell lines.**

5. **To identify the role of small Rho GTPase signaling during HSV cell-cell spread, we examined the activation of small Rho GTPases during HSV infection, and the effect expression of dominant negative and constitutively active forms of Rho GTPases had on fusion.**

III.   OVERVIEW

Herpes simplex encephalitis

HSE is the most common fatal sporadic encephalitis in humans, which has an extremely high mortality rate when antiviral treatment is not used or not effective (e.g. up to 70% in newborns) with fewer than 3% of survivors returning to normal function *[1]*. With the advent of safe and effective antiviral therapy (acyclovir) for treating HSE, significant improvements in mortality and morbidity have been achieved *[2]*. The primary effective therapy used against herpesviruses is the nucleoside analogue acyclovir *[3]*. Unfortunately, acyclovir therapy only marginally decreases the neurological impairment suffered among HSE survivors *[2]*. These neurological sequelae include major defects of memory and affect, which are often incompatible with personal and social rehabilitation. Neurological pathology and behavioral defects similar to those observed in humans have been seen in rats following experimental HSE *[4]*.

HSV-1 is responsible for most cases of HSE in both children and adults, while HSV-2 primarily induces HSE in most neonatal cases and occasional adult cases. Neonatal HSV infection is usually acquired at birth, although a few infants have had findings suggestive of intrauterine infection. Intrauterine HSV infection can occur as a consequence of either primary or recurrent maternal infection and has severe consequences for the fetus. Neonatal HSE can involve any, and often multiple, parts of the brain, in contrast with the typical temporal lobe predilection seen with HSE that has onset beyond the neonatal period *[5]*. Pathological manifestations of intrauterine HSE are cortical atrophy, hydrocephalus, microcephaly, periventricular leukomalacia, and microphthalmia *[6]*. These brain malformations are suggested to be the result of direct viral destruction of the CNS.

Nectin-1 serves as the primary HSV receptor in the brain

Nectin is a Ca^{2+}-independent cell–cell adhesion molecule that belongs to the immunoglobulin superfamily *[7-11]*. Nectin-1 was originally isolated as one of the poliovirus-receptor-related proteins and named PRR-1 *[12]*.

The nectin family consists of at least four members: nectin-1, nectin-2, nectin-3, and nectin-4, all of which, except nectin-4, have two or three splice variants: nectin-1 (α, β, γ), nectin-2 (α, δ), nectin-3 (α, β, γ) *[9, 11-16]*. All nectin members, except nectin-1γ, have an extracellular region containing three Ig-like domains, a single transmembrane region, and a cytoplasmic region *[8, 10, 11]*. Nectin-1α has a conserved four residue motif (Glu/Ala-X-Tyr-Val) that binds the PDZ domain of afadin, which is directly associated with F-actin bundles, at its cytoplasmic C-termini *[9, 11, 16, 17]*.

Nectin-1 forms both homo-cis-dimers and homo-trans dimers *[8, 10, 11]*. Furthermore, it forms hetero-trans-dimers with nectin-3. Hetero-trans-dimer formation is stronger than that of homo-trans-dimers *[11]*. Nectin–afadin system organizes adherens junctions cooperatively with the cadherin–catenin system in neural *[18-20]* and nonneuronal cells *[8, 16, 21]*. These nectin-1 trans-interactions can activate small Rho GTPases *[22]*. The formation of adherens junctions is suppressed by inhibition of Rho *[23, 24]*. Nectin-1 is involved in the formation of synapses between the mossy fiber terminals and the dendrites of pyramidal cells in the CA3 area of the adult mouse hippocampus *[18-20]*. Nectin-1 takes part in recruitment of intercellular junctions that play essential roles in various cellular processes, including morphogenesis, differentiation, proliferation, and migration *[25-27]*.

Human nectin-1 also serves as an entry receptor for all α-herpes viruses tested so far, including herpes simplex virus (HSV) type 1, HSV type 2 and pseudorabies virus *[28, 29]*.

Herpes simplex virus

Herpes simplex virus type–1 and –2 (HSV-1 and HSV-2) are neurotropic viruses belonging to a subfamily of herpesviruses known as alphaherpesviruses.

While usual manifestations of HSV disease are mucocutaneous lesions, HSV infection can also spread to the central nervous system (CNS) and cause life threatening illnesses, including meningitis and herpes simplex encephalitis (HSE). HSV establishes latent infection of neurons in the sensory ganglia and causes recurrent lesions at the sites of primary infection *[30, 31]*. Intercellular spreading of HSV significantly contributes to its pathogenesis and the development of disease.

The underlying mechanisms of HSV entry and spread using nectin-1 are not fully understood, but at least four HSV glycoproteins (gD, gB, and the gH-gL heterodimer) are required for HSV-1 entry and spread [28, 29].Recombinant gD binds nectin-1 in host cells and inhibits HSV-1 infection, indicating gD specifically interacts with nectin-1. The first Ig-like domain of nectin-1 is sufficient for gD binding and entry of HSV-1, while the second and third Ig-like domains increase the efficiency of entry [14]. Both HSV-1 and -2 use nectin-1 as a receptor. Both gD-1 and gD-2 have a similar affinity for nectin-1 [32, 33]. HSV-1 and HSV-2 can use an additional receptor, HVEM. [34]. HSV-2 may also bind another unidentified receptor [35]. In addition, some, but not all, HSV-1 isolates from the CNS were able to infect cells expressing nectin-2, a receptor not used by wild-type HSV lab strains [36, 37]. In addition to these protein entry mediators, 3-O-sulfated heparin sulfate serves as a gD receptor specifically for HSV-1 [38].

Presence of HSV-1 DNA has been reported in several neuronal disease, including schizophrenia [39] and Alzheimer disease's [40]. HSVs are candidate infectious agents that may contribute to the psychopathology and aetiology of various neurological diseases.

Oxidative stress and damage

Oxidative stress occurs when balance between pro-oxidant stimuli and the antioxidant mechanism is displaced to favor the pro-oxidant stimuli. Reactive oxygen species (ROS) can be induced within cells by redox reactions associated with normal physiologic processes or enzymatic and nonenzymatic mechanisms associated with pathologic processes. ROS include free radicals, such as suproxide (O_2^-,) and hydroxyl (OH) that contain one or more unpaired electrons, and reactive nonradicals, like hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO_2$) that are oxidizing agents or easily converted into radicals [41]. ROS readily enter cells and interact with macromolecules, especially unsaturated membrane lipids and nucleic acids. ROS are generated in inflamed tissue by infiltrating NADPH expressing phagocytic cells [42]. Free radicals can cause lipid peroxidation, an autocatalytic process that damages lipid containing structures and yields reactive by-products, primarily 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA). HNE and MDA are electrophilic species that can

covalently modify and damage cellular macromolecules. At physiologic concentrations, HNE and MDA are potent regulators of cell growth and differentiation, affecting both cellular transcription and cell cycle progression *[43-45]*.

Oxidative cellular injury can cause cellular dysfunction and, when severe, cause cell death. Studies suggest viral-induced oxidative damage induced by virus infection can be purely deleterious to the host. ROS and secondary lipid peroxidation products, like HNE and MDA, may affect viral replication by modifying the activation state of cells' regulation of host inflammatory and immune responses, and by causing oxidative damage to host tissues and viral components *[46-49]*. In addition, ROS has also been shown to disrupt the blood brain barrier, using intercellular signaling pathways *[50]*. This leads to invasion of immune cells into the CNS and contributes to neuroinflammatory diseases. One of those pathways is believed to be Rho signaling.

Small GTPases

Rho GTPases are molecular switches that cycle between an inactive guanosine diphosphate (GDP)–bound state and active GTP-bound state. In the active GTP-bound conformation, each Rho GTPase binds to a subset of downstream effectors, which in turn activate or inactivate downstream proteins to generate the appropriate outcome *[51]*. Effectors for RhoA include Rho kinase (ROCK), citron, and mDia. Twenty different Rho GTPases, divided into five classes, regulate various aspects of cytoskeletal dynamics.Rho signaling can be activated by a number of cellular receptors, including nectin-1 *[22]*.

Cycling between the active and inactive state is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) *[52]*. Activation of ubiquitously expressed Rho GTPase family members Rho, Rac, and Cdc42 induces rearrangements of the actin cytoskeleton that result in formation of actin stress fibers, membrane ruffling or lamellipodia, and protrusive filopodia, respectively *[51] [53]*. Small GTPases play a significant role in a wide variety of cellular processes, including proliferation, differentiation, microtubule stabilization,

endocytosis, vesicle trafficking, cytoplasmic transport, and gene expression *[54]*
[55].

Rho signaling is also involved in the disruption tight junctions, leading to impairment of the blood brain barrier *[50]*. This results in the migration of immune cells into the CNS, which can contribute to the development of neuroinflammatory diseases.

Cell migration in the CNS during the brain development

Cortical neurons form at the same time in the ventricular zone (VZ), and migrate to the same cortical layer. Preplate cells (PP) are the youngest neurons. They migrate from the VZ to the pial surface and form the preplate layer. The intermediate zone (IZ) then settles between the ventricular and preplate layers, and the compact cortical plate (CP) develops. Postmitotic cells of the cortical plate cross the intermediate zone during migration. The layer above the cortical plate is the marginal zone, which forms the I. layer of the cortex. The layer below the cortical plate is the subplate zone (Fig.1). This pattern forms the typical cortex organization: "inside first-outside last" *[56, 57]*.

It is known that two types of cell migration routes exist: radial and tangential migration. It has been known since 1970 that postmitotic neurons migrate radially
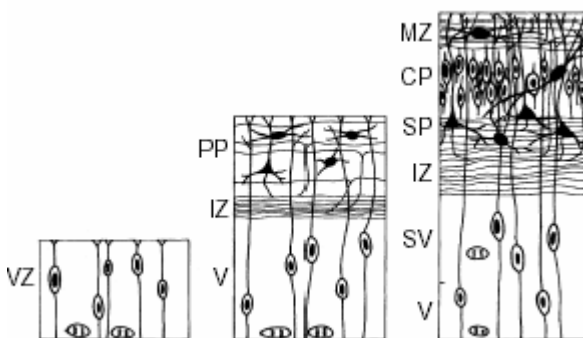

Figure 1. The organization of the cortex during embrional development *(McConnell 1995)*

away from the germinal zones to the cerebral cortex where they form mature neurons along a discrete pathway. Neurons are generated within the proliferative layers, which includes the ventricular and subventricular zones. The ventricular zone is adjacent to the cerebral ventricular system, where the majority of cortical neurons originate. The subventricular zone appears only during a given part of the development. This second focus of proliferation is found in a scattered pattern, with one part lining along the ventricle and the other in the subventricular intermediate zone. Glia cells mainly originate from this proliferative zone.

For radial migration to occur, intimate contact between migrating neuron and guiding glia cells is required. The radial glia phenotype is indicated by Cajal-Retzius (CR) cells located in the first layer of marginal zones. Elimination of CR cells in newborn mice results in impaired neocortical lamination parallel with radial glia dedifferentiation *[58]*. Failed cortical development in humans leads to permanent neurological disease, such as mycrogyria and ectopy, which is escorted by mental retardation *[58, 59]*. CR is the main reelin protein-expressing cell population *[60]*. Reelin is a stop signal for migrating neurons. In reeler mutants, degradation of the radial glia scaffold has been observed **.***[61]*. The role of CR cells in regulation of radial cell migration is temporary and limited to well-defined parts of brain development. Except for some brain regions (e.g. DG), these cells can disappear, which is caused by changing sets of extracellular proteins, neuronal activity, or the presence of different growth factors.

Radial migration is supplemented by rapid tangential migration. This migration type is not limited to short distances because it does not require guiding glia cells, and because different cell populations besides post mitotic cells are involved. Two types of tangential migration have been reported: neurophil migration, where axons serve as a guiding pathway, and homophile migration, where migrating cells form a chain by connecting with each other during migration. These special chains of migrating neurons were first recorded in adult mice brain and named rostralis migration streams (RMS) *[62]*. RMS is a polysialic acid form of the neural cell adhesion molecule (PSA-NCAM)–immunopositive pathway from the subventricular zone to the core of the ipsilateral olfactory bulb.

Although well-documented *[63-65],* interaction of the radial and tangential migratory systems during brain development is still unclear.

IV.   MATERIALS AND METHODS

1. ANIMALS AND PREPARATIONS

Experimental procedures used followed the protocol for animal care approved by both the Hungarian Health Committee (1998) and the European Communities Council Directives (86/609/EEC). Inbred BALB/c mice were raised with access to water and food pellets (Altromin) *ad libitum*. Females were mated overnight and checked for vaginal plugs the following morning. A positive vaginal plug was considered the day of conception, embryonic day 0 (E0). Birth usually occurred on day E19 or E20. To normalize the ages of the experimental animals, E20 was considered equivalent to postnatal day 0 (P0). Pregnant mice were checked twice daily to determine time of birth for litters. Pups and adults were deeply anaesthetized (ketamine 10.0mg/100g and xylazine 0.8mg/100g body weight intraperitoneal), perfused transcardially for 5 to 10min with ice-cold phosphate-buffered saline (PBS; 0.1M, pH7.3), and fixed in 0.4% paraformaldehyde (PFA) for the same amount of time. After, brains were soaked in the same fixative overnight at 4^oC. For cryoprotection, sections were first placed in 20% sucrose solution for 2h, then 25% sucrose solution overnight, and were then embedded in OCT (Optimum Cutting Temperature media) and frozen with liquid nitrogen. They were cut on a cryostat at 15 to 20μm in the coronal plane.

2. HUMAN TISSUES

Sections of formalin-fixed, paraffin-embedded archival normal adult human tissues and human fetal tissues from autopsies performed on fetuses with a clinically estimated gestational age of 20 to 22 weeks were used in this study. Use of these tissues was approved by the Institutional Review Board of the University of Illinois at Chicago.

3. CELLS AND VIRUS

Cells

Mouse embryonal carcinoma P19 cells (ATCC, CRL-1825) were maintained at 37^oC in 5% CO_2 using growth medium consisting of 90% α-minimum essential medium (Gibco), 7.5% calf serum (Gibco), and 2.5% fetal bovine serum (FBS)

(Gibco) [66]. Vero cells were maintained at 37°C in 5% CO_2 in Dubelco's Modified Essential Media (DMEM) (BioWhittaker) supplemented with 10% FBS (Gibco), 4mM L-glutamine, and 110mg/L sodium pyruvate. All growth media was supplemented by 100U penicillin-streptomycin (MP Biomedicals Inc) to provide final concentration of 50U/ml and 50µg/ml, respectively.

Differentiation of P19 cells into neural cell cultures (P19N)

P19 cells were differentiated by treatment with retinoic acid according to established protocols *[66, 67]*. P19 cells were grown in culture flasks until confluent and transferred into Petri dishes. Retinoic acid dissolved in ethanol (Sigma; 10–5µM final concentration) was added to the cells. Retinoic acid was added again to the cells each time the culture medium was changed over the course of the next 5 to 7 days. Differentiated (P19N) cells were transferred to 75cm^2 flasks or 96-well plates, and retinoic acid treatment continued until the cells were used in the assay.

Virus

HSV-1 wild-type strain F was obtained from Dr. B.He (University of Illinois at Chicago). Virus stocks of HSV-1 wild type strain KOS (available in our laboratory) and strain F were prepared and titered using Vero cells as previously described *[68]*. Heat (65°C for 30 min) and ultraviolet (UV) light inactivation of viruses was performed as previously described *[68]*. For mock infected controls, cells extracts were prepared from uninfected Vero cell cultures following the same protocol used for the preparation of virus stocks from HSV-1 infected Vero cell cultures.

4. IMMUNHISTOCHEMISTRY

Primary antibodies used in this study and their dilutions were: rat monoclonal anti-mouse nectin- 1 (clone 48-12) (courtesy of Dr. Y.Takai, Osaka University), 1:2; rabbit anti-human nectin-1 (R166), 1:1000. Rat anti-mouse nectin-1 monoclonal antibody (clone 48-12) was prepared and specificity was confirmed as described by Takahashi [16]. This antibody, which recognizes the extracellular domain of mouse nectin-1, shows reactivity against mouse and rat neurons *[18]*. Rabbit anti-human nectin-1 polyclonal serum (R166) (courtesy of Prof. R.J.

Eisenberg, G.H. Cohen and C. Krummenacher, University of Pennsylvania) recognizes the ectodomain, and shows reactivity against nectin-1 in human and rat neurons *[69, 70]*. The following biotinylated secondary antibodies were used: anti-rat IgG (H+L), mouse adsorbed, made in rabbit (Vector Labs;), AMCA-conjugated anti-rat IgG (H–L) made in donkey (Jackson Lab;), anti-rabbit IgG (H+L), made in goat (Vector Labs). All were diluted 1:200. Immunolabeling was revealed using the avidin-biotin-HRP (horseradish peroxidase) method (VECTASTAIN Elite ABC; Vector Labs) (except of AMCA conj. sections). Sections were washed in 0.01M PBS, pH7.4, reincubated for 60min at room temperature (RT) with blocking serum (10% normal goat serum, 0.01% Triton X-100 in 0.01M PBS, pH7.4,) and incubated overnight at 4ºC with primary antibody diluted in the same blocking serum. Next, sections were washed with PBS and incubated for 1 to 2h at RT with secondary antibody diluted in blocking serum. Sections were rinsed with PBS and exposed to avidin-biotin-HRP complex for 30 to 40min (except of AMCA conj. sections). HRP was visualized with 3,3 diaminobenzidine and AMCA conj. samples were visualized with fluorescent microscopy. Sections were mounted on Superfrost microscope slides (Fisher), dehydrated, and coverslipped with Entellan (Merck). For control sections, either the primary antibody was omitted or preimmun sera were used. No labeling was found in the controls. Slides were processed digitally (Olympus BX51, DP70, and Olympus DP Manager).

Immunohistochemistry in tissue culture was performed by growing P19N cells on chamber slides and inoculating with 1 PFU/cell of HSV-1 KOS or mock infecting with virus free Vero cell extract. Cells were incubated with virus or virus free extract for 1h at 37ºC with occasional gentle agitation, washed twice in sterile PBS, and incubated for 24h in fresh medium. Media was discarded, and cultures were washed twice in sterile PBS and fixed in 4% PFA for 2h. HSV-1 antigens were detected using a 1:1000 dilution of rabbit HSV-1–specific antiserum (DAKO). Incubation with primary antibody for 32 min at 43ºC was followed by addition of biotinylated anti-rabbit IgG secondary antibody, avidin-HRP, and finally 3,3-diaminobenzidine tetrahydrochloride (0.04%) in 0.05 M Tris-HCL (pH7.4) and 0.025% H_2O_2 as a chromogen (Ventana Medical Systems). Before staining, binding of secondary antibodies and conjugates was blocked by appropriate reagents provided by the manufacturer.

5. WESTERN BLOT

Western Blot Analysis—Protein (20mg applied per lane) was separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membrane (Bioscience, PROTRAN, Whatman) using a semidry transfer apparatus (Bio-Rad). For blocking nonspecific binding, membranes were incubated 1h at RT in 5% nonfat dry milk dissolved in TTBS (10mM Tris-HCl (pH7.5), 150 mM NaCl, and 0.1% Tween 20). Next, membranes were probed for 2h at RT with mouse anti-RhoA monoclonal antibody (Santa Cruz Biotechnology) diluted in TTBS (1:500). After extensive washing in TTBS, the membrane was incubated for 1h with HRP-conjugated anti-mouse secondary antibody (Jackson, 1:10000). Chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce) was used to visualize protein bands which were exposed on KODAKBioMax MRfilm.

RhoA Activation Assays (GST-tagged Rho protein pull-down assay)–A pull-down assay was used to isolate active GTP-bound RhoA. Active RhoA was purified using Rhotekin-RBD-GST beads (Cytoskeleton), which specifically bind only to active RhoA. Total protein was incubated with beads for 1h at 4℃ on a rotator. Purified RhoA was detected by Western blotting. Control samples were treated with GTPγS (positive control) and baseline activation of RhoA served as an internal control in every pull-down assay.

6. HSV-1 INFECTION

P19N and Vero cells were washed once with sterile PBS (pH7.4). Cells were then inoculated with either (i) sterile PBS, (ii) sterile PBS containing the appropriate amount of HSV-1 stock to reach the intended multiplicity of infection (MOI), or (iii) sterile PBS containing cell extract prepared from uninfected Vero cell cultures following the same protocol used for the preparation of virus stocks from HSV-1 infected Vero cell cultures (mock infection). Flasks were incubated at 37ºC for 1h with occasional gentle agitation. Next, inocula used for infection or mock infection were removed and fresh culture medium was added.

7. PLAQUE ASSAY

Frozen culture supernatant was defrosted and dilutions were prepared (10^1-10^4) from virus containing supernatant and control samples. Diluted supernatant was introduced to the cells in duplicate for 1h with occasional gentle agitation at

$37°C$ in 5% CO_2. After, 1mL 0.5% methylcellulose in growth medium supplemented with 10% FBS (Gibco) and 100U penicillin-streptomycin (MP Biomedicals Inc) was added to each well. Following incubation at $37°C$ in 5% CO_2 for 3 days, plaques were counted.

8. SYNCYTIA ASSAY

Syncytia were visualized using either Giemsa staining or fluorescence. For Giemsa staining, target and effector cells were mixed in a 1:1 ratio for 72h. Cells were fixed in absolute methanol for 20min, washed in PBS, and stained with freshly prepared Giemsa stain (stock solution diluted 1:20 in water) for 20min. Stained cells were decolorized in 95% ethanol for a few seconds and rinsed in water. Giemsa stock solution was prepared by dissolving 0.5g of Giemsa powder in 33ml of glycerine in a water bath ($55-60°C$) for 90 min. After powder was dissolved, 33ml of absolute methanol was added. Stock solution was stored at RT.

In the fluorescence assay, both target and effector cells were additionally transfected with green fluorescent protein (GFP). At 72h post-mixing, syncytia formation was observed using a fluorescent light microscope (Leica)

9. MOLECULAR BIOLOGY

Detection of reactive oxygen radicals

P19N cells suspended in fresh culture medium were added to 96-well plates (BD Falcon) and incubated overnight at $37°C$ in 5% CO_2. Cells were washed once with PBS to remove unattached cells and serum. The wells were either inoculated with HSV-1, mock infected, or exposed to sterile PBS. Following a 1h incubation period at $37°C$, cells were washed once with PBS, fresh culture medium without phenol red was then added to each well, and plates were incubated at $37°C$ in 5% CO_2 for selected time periods. The wells were then washed once with PBS again. Hydroxyphenyl fluorescein (HPF; Molecular Probes; $5\mu M$ final concentration) in fresh culture medium without phenol red was added to each well to detect presence of reactive oxygen species (ROS) [71]. Nonfluorescent HPF becomes fluorescent in presence of ROS, particularly hydroxyl radicals. Plates were incubated at $37°C$ in 5% CO_2 for 20 to 60min. Wells were washed once with PBS

to remove excess HPF. Fresh PBS was added to each well and fluorescent intensity was measured using a Tecan GENios Pro plate reader with fluorescent excitation and emission maxima of 490nm and 515nm, respectively.

Measurement of lipid peroxidation with Bioxytech LPO-586 assay

These experiments were performed in 75-cm^2 tissue culture flasks (BD Falcon). P19N cells were washed once with PBS to remove unattached cells and serum. Flasks were either exposed to (i) HSV-1 diluted in PBS, (ii) cell extract diluted in PBS (mock infection), or (iii) PBS alone. Cells were washed once with PBS after a 1h incubation period, and fresh growth medium without phenol red was added. Aliquots of culture supernatant were taken from each flask at selected times. Butylated hydroxytoluene (ICN Biomedicals; 5mM final concentration) was added to each aliquot to prevent further oxidation, and the samples were centrifuged (3000 × g, 10min, 4ºC). The aqueous portions were frozen and stored at −80ºC until ready for analysis. Combined levels of MDA and 4-HAE in the samples were determined using the Bioxytech LPO-g586 colorimetric assay (Oxis International) according to the manufacturer's instructions. Detected levels of MDA and 4-HAE were normalized to the amounts of total protein measured in the samples. Total protein in each sample was determined using the Coomassie Plus Protein Assay Kit (Pierce) according to the manufacturer's instructions. All measurements were made using a Biomate 3 spectrophotometer.

HNE treatment

Different concentrations of HNE were freshly prepared in 100% ethanol before addition to the samples. Equal distribution of the vehicle (100% ethanol) was used in all samples, including the controls which did not get HNE, at a final concentration of 0.05%. Cells were treated with 0, 5, or 50µM HNE for 0.5 or 1h in low sera containing culture medium at 37℃ in 5% CO_2. After incubation, cells were washed once and infected with HSV-1 virus abruptly.

Antioxidant assay

The 10mM stock solution of ebselen was freshly prepared in DMSO before addition to the samples. P19N cells were treated with 0, 5, 10, or 25µM (final

conc.) ebselen, which was added to culture media for 1h prior to HSV-1 infection, and following the 1h virus infection, for 24h at 37℃ in 5% CO_2. After, aliquots of the culture supernatant were taken from each well. Samples were frozen and stored at -80℃ until ready for use in the plaque a ssay.

Cell-cell fusion β-Galactosidase reporter assay

Wild type Chinese Hamster Ovary (CHO-K1) cells were split into two populations. Target cells were transfected with nectin-1 and ω-peptide plasmid of the β-Galactosidase gene. Effector cells were transfected with virus glycoproteins necessary for cell-cell fusion (gB,gD,gH,gL) and the α-peptide of β-Galactosidase gene. At 16h after transfection, target and effector cells were mixed in 1:1 ratio, transferred to 96 well dishes, and incubated at 37°C. After 48h, cells were lysed in 0.5% Nonidet P-40, chlorophenol red-β-D-galactopyranoside (Roche Diagnostics) was added, and β-galactosidase activity was determined by absorbance at 560nm using a microtiter plate reader (Dynatech).

V.   RESULTS

Herpes simplex virus type 1 (HSV-1) is the primary causative agent of sporadic encephalitis. In adult human, postmortem, computed tomography (CT), and magnetic resonance imaging (MRI) studies showed that HSE affected particularly CNS structures implicated with cognitive functioning, including the amygdale, hippocampus, and related limbic areas. Different explanations exist for the site specificity of HSE that believe it leans on the routes of access of HSV to the CNS, but none of them consider how HSV spread respects the boundaries of an anatomical, developmental and functional unit: the limbic system. Nectin-1 is the primary receptor for HSV in the nervous system. Though it is known nectin-1 is expressed in the brain, its spatiotemporal expression pattern, as of now, is unknown. Determining nectin-1 expression in the brain, especially during development, could serve to elucidate this paradigm.

Nectin-1 expression selectively increased in limbic regions and concomitantly decreased in association areas during postnatal brain development

To determine whether changes in nectin-1 distribution during the postnatal brain development are consistent with the pathobiological dichotomy of HSE, brains of newborn and adult BALB/c mice were immunostained to detect nectin-1 protein expression. Anti-nectin-1 monoclonal antibody was used to detect the distribution of the receptor throughout the brains of mice. In the newborn mouse brain, the most intense immunoreactivity of nectin-1 was observed as a punctate pattern along the leading and lagging axonal processes and on cell bodies of neurons in the interconnecting structures of the hemispheres (corpus callosum, hippocampal, and anterior commissures) and in the related cortical areas (Fig. 2A, D). These cells have the distinct phenotype of migrating neurons. Subgroups of nectin−1−positive neurons diverge and follow distinct migratory pathways (radial migration along the glial pathway and tangential migration in the intermediate zone). The near-midline sagittal section of this region also revealed an extensive nectin−1−negative band between cells lining the ventricle and postmitotic neurons destined to the cortical layers in the intermediate zone. This

zone is anatomically equivalent to the subventricular zone (SVZ). In the adult brain, however, expression in specific limbic-related regions, such as the Ammon's horn, dentate gyrus, frontal association cortex, olfactory bulb, and the RMS (rostral migratory stream) remained high, whereas, expression in nonlimbic regions decreased to low levels (Fig. 2B, F). It may be noted that the nectin–1–negative SVZ of the newborn mouse disappears in the adult stage, and nectin–1 staining is lacking in the interhemispheric connecting structures (CC, CH, AC) (Fig. 2B). The loss of the nectin–1–negative SVZ in adulthood hints at a critical role of this definite region in neural cell generation during brain development.

Figure 2 Nectin-1 immunostaining of paramedian sagittal sections of newborn (A) and adult (B) murine brains
A: A prominent nectin-1 signal can be detected in the interconnecting structures of the hemispheres (CC, CH, AC). Arrays of nectin-1–positive radially oriented migrating neurons can be seen in the cerebral cortical wall. A fainter, circumscribed nectin-1 signal was detected in the RMSs and RMSc, which contains neuroblasts migrating from the SVZ to the OB. Strong nectin-1 expression was detected in the zone of the corpus callosum to the external layers of the developing olfactory bulb, which we name the callosal migratory stream (CMS). **B**: In the adult mouse, marked nectin-1 immunoreactivity was observed in the hippocampal formation (CA, DG) and in the RMS. **C, E**: Nectin-1 labeling in the anterior frontal cortex. The black arrowheads indicate radial expansion of cortical neurons in the newborn frontal pole. In the adult anterior frontal lobe mature pyramidal neurons with similar distribution express nectin-1 (white arrowheads). **D, F**: No relevant labeling was found in the control sections treated with preimmun sera. Areas surrounded by rectangles indicate the control regions for higher magnification in **C** and **E**. The scale bars on the photomicrographs **A**, **C**, and **E** denote1 mm and 500 µm on **D, F**.
AC, anterior commissure; CA, cornu ammonis; CC, corpus callosum; CH, commissura hippocampi; CMS, callosal migratory stream; CTX, cerebral cortex; DG, dentate gyrus; FrA, frontal association cortex; OB, olfactory bulb; RMSs, rostral migratory stream, striatal part; RMSc, rostral migratory stream, cortical part; SVZ, subventricular zone.

The RMS and the callosal migratory stream (CMS): a different spatial and temporal pattern of nectin-1 expression during development

The RMS, described by Rousselot et al.*[62]*, is a polysialic acid form of neural cell adhesion molecule (PSA-NCAM)–immunopositive pathway from the subventricular zone to the core of the ipsilateral olfactory bulb. These special chains of migrating neurons were first recorded in adult mice, but further studies extended this notion to a similar phenomenon in every developmental stage. Here we identified two similar, but fundamentally different, migratory systems of the olfactory bulb based on nectin-1 expression. In the adult mouse, nectin-1 immunoreactivity was detected as long arrays of cells lined up in chains (Fig. 2B). These arrays of cells crossed the anterior olfactory nucleus and entered the olfactory bulb where they expanded radially from the subventricular zone surrounding the lateral ventricle. This result indicates that the RMS is located under the corpus callosum and is not a continuation of it. In contrast, nectin-1 immunopositivity of the same area of the neonatal mouse brain was weak (Fig. 2A). Chains of small cells displaying fusiform morphology could not be detected. Nevertheless, nectin-1 expression was strong in migrating cells of the corpus callosum and the intermediate zone of the developing cerebral cortex (Fig. 3A). These arrays of migrating cells move along a RMS-like pathway, but in a slightly
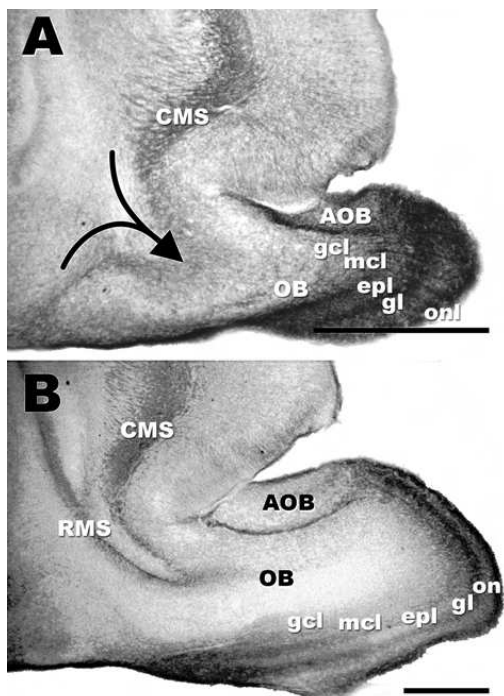

Figure 3
Comparative analysis of nectin-1 distribution in the developing olfactory system of the newborn mouse and rat
A: The nectin-1 immunopositivity of the RMS was only weak on **P0** in the **mouse**. Chains of small cells displaying fusiform morphology could not be detected. Nectin-1 was detected in the migrating cells of the corpus callosum and the intermediate zone of the developing cerebral cortex. These arrays of migrating cells move along a RMS-like pathway (*black arrow*). We name this pathway the callosal migratory stream (CMS). **B**: **In the newborn rat brain**, unlike the same area in mice on P0, we detected marked nectin-1 immunoreactivity in both the RMS and the CMS. Note the high expression level of nectin-1 in both species. Scale bars denote 1 mm.
AOB, accessory olfactory bulb; epl, external plexiform layer; gcl, granule cell layer; gl, glomerular layer; mcl, mitral cell layer; OB, olfactory bulb; onl, olfactorynerve layer.

dorsal layer (in the zone of the corpus callosum to the external layers of the developing olfactory bulb) and a step earlier in development (postnatal days 0 to 4; P0 to P4) than the real RMS (P7 to adult). Accordingly, we named this pathway the CMS (callosal migratory stream). To exclude the possibility of antibody cross-reactive staining, we determined whether similar dual migratory pathways (RMS and CMS) are present in the newborn rat olfactory system. To do so, we used polyclonal anti-human nectin-1 antibody known to react with rat nectin-1 due to high conservation of nectin-1 between species *[72-74]*. In all of the processed brains, both migratory streams were detected, entering the developing olfactory bulb in parallel. Unlike the same area of the mouse on P0, here we detected marked nectin-1 immunoreactivity in both the RMS and CMS (Fig. 3B).

Nectin-1 is involved in perinatal telencephalic commissuration in the murine brain

A distinction between adult and neonatal HSE is that the former is essentially unilateral whereas the latter is mostly bilateral. This implies that the corpus callosum (CC) should be a key determinant of unilateral versus bilateral spread of
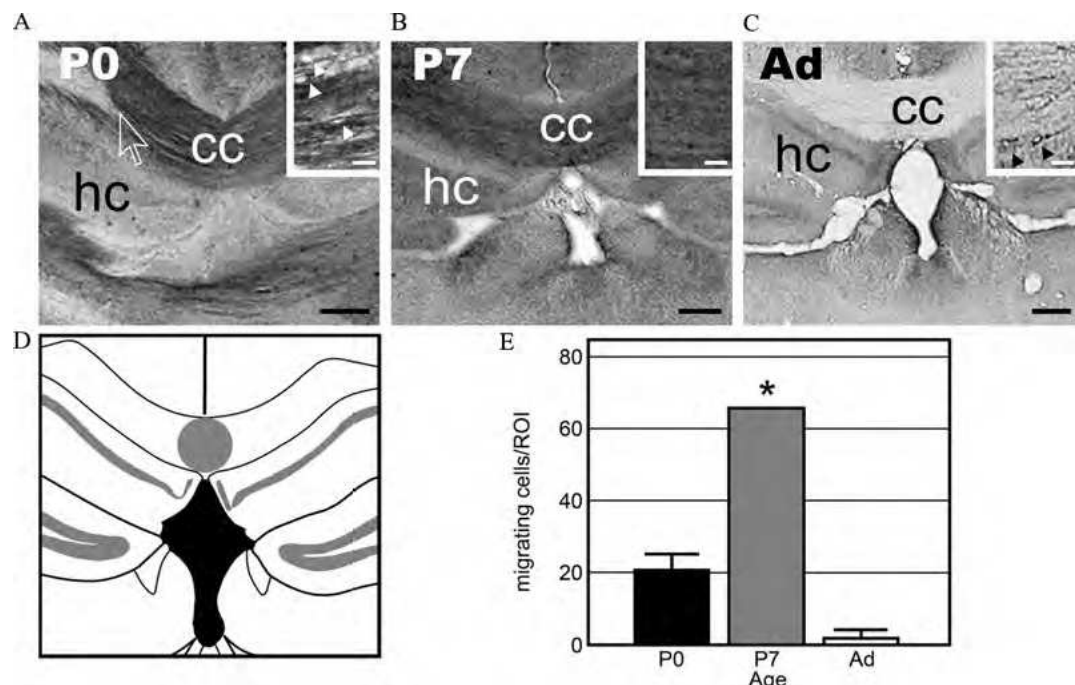

Figure 4 Developmental changes in nectin-1 distribution in the interhemispheric connections.
A: Coronal section of the brain of newborn mouse (P0). Insert shows the fusiform, spindle-shaped cells (white arrowheads). A nectin-1–negative band between the corpus callosum and the commissura hippocampi (empty arrowhead) **B:** On P7, the whole corpus callosum stained positive for nectin-1 (Insert) the fasciculation of transcallosal axon bundles was not distinguishable. **C:** Coronal section of adult mouse brain. (Insert) Nectin-1 labeled few cells with a fusiform-ovoid perikaryon and prominent, long leading processes along the nectin-1–negative callosal bundles. **D:** Schematic drawing of the region of interest. The gray circle represents the region of interest (ROI) used for counting callosal migratory cells. **E:** Histograms illustrating the age-related numbers (±SEM) of nectin-1–positive migrating cells in the ROI. *Only estimated data; The scale bar denotes 200 µm, and in the insert 20 µm.
cc, corpus callosum, hc, hippocampus.

HSE. Therefore, we made a detailed examination of the developing corpus callosum. In the coronal sections of the brains of newborn mice, we detected massive nectin-1–positive callosal bundles and migrating neurons (Fig. 4A, E). Numerous fusiform, spindle-shaped cells displayed a very thin perikaryon with long leading processes all along the callosal fibers (insert in Fig. 4A). At P0, the corpus callosum was not fully filled with these fascicles and migrating elements, and there was a nectin-1–negative band between the corpus callosum and commissura hippocampi (Fig. 4A empty arrowhead). The medial commissural pathways were localized in the upper part and the lateral interhemispheric pathways in the lower part of the corpus callosum. In the corpus callosum on P7, no hiatus of nectin-1 expression was detected between the commissura hippocampi and the corpus callosum (Fig. 4B). The whole corpus callosum was homogeneous for nectin-1 staining, and the fasciculation of transcallosal axonbundles could no longer be distinguished. The nectin-1 signal labeled the callosal area so densely that we could only estimate the number of migrating cells (Fig. 4B insert, E). In the adult corpus callosum, however, only faint to zero nectin-1 expression was detected. Nectin-1 labeled only a few cells displaying a fusiform-ovoid perikaryon and prominent, long leading processes along nectin-1–negative callosal bundles (Fig. 4E). Nectin-1 appears to be expressed in the CC of the developing mouse brain at stages when HSE shows bilateral dissemination, whereas, it is absent in adult CC when HSE is mostly unilateral.

Nectin-1 expression in newborn murine and fetal human neocortex

Next, we compared human and mouse samples to identify similar expression patterns of nectin-1 in both developing nervous systems, especially in the hippocampus and the cortex.

In the newborn mouse cortex, the highest nectin-1 signal was associated with the cerebral cortex (Fig. 5A). The transient strata: ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (FL), and subplate (SP), had highly specialized nectin-1 staining pattern in the newborn murine brain. Nectin-1 immunoreactivity was prominent in the upper tiers of the cortical plate just below the marginal zone with nectin-1-positive neurons having the morphologic appearance of Cajal-Retzius cells (Fig. 5B). Radially orientated apical dendrites of

nectin-1-positive neurons indicated a predominant pyramidal phenotype in layers II/III. Nectin-1 was also expressed in migrating cells in the intermediate zone and neurons in the cortical plate, and cells of the ventricular zone. The intermediate zone is the region traversed by the early afferents and efferents to the cerebral cortex, and through which, immature neurons have to migrate enroute to the cortical plate. Nectin-1 protein was observed in cell somata and processes, and in
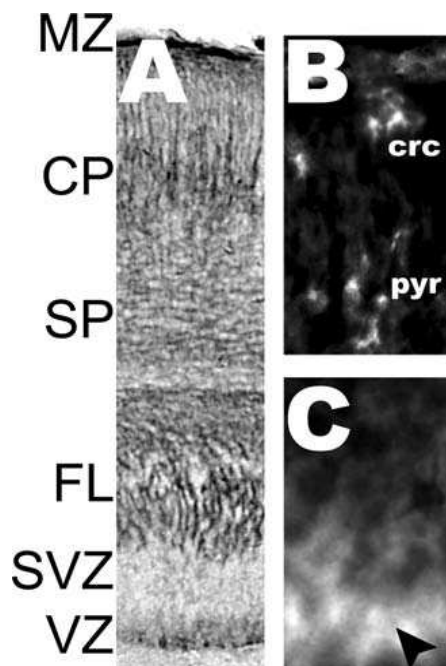

Figure 5
Nectin-1–specific immunolabeling in the newborn mouse cortex (**A**) Nectin-1 was expressed in the migrating cells in the intermediate zone and in neurons in the cortical plate, as well as in cells of the ventricular zone. (**B**) nectin-1-specific immunofluorescent signal was prominent in the upper tiers of the cortical plate just below the marginal zone with nectin-1-positive Cajal-Retzius cells (crc) and radially orientated nectin-1-positive pyramidal neurons (pyr). (**C**) Cloud-like nectin-1 immunostaining pattern around radial glia-like cells in the ventricular zone (*arrow*) with nectin-1-negative stellate-shape in the upper part. MZ marginal zone, CP cortical plate, SP subplate, FL intermediate zone, SVZ subventricular zone, VZ ventricular zone

the horizontal fiber tracts characteristic of tangential dispersion of migratory cells within the intermediate zone (Fig. 5A). Nectin-1 protein immunosignal was hardly detected in a subset of cells in the VZ. These cells had large oval somata and lay close to the ventricular surface. Nectin-1 immunoreactivity was preferentially concentrated at the ventricular pole of the cytoplasm and in the endfeet lining the ventricle (Fig. 5C). Despite the cloud-like nectin-1 immunostaining pattern around these cells, it was assumed the position and shape of these nectin-1-positive cells are characteristic to radial glia like cells. Nectin-1 concentration in ventricular endfeet and initial portions of radial processes of ventricular-zone cells points to a possible involvement of nectin-1 in guidance of neuronal migration.

In the human fetal neocortex, nectin-1 was strongly expressed in the leptomeninges. In the most superficial, marginal zone of the neocortex, nectin-1-positive neurons with the morphologic appearance of Cajal-Retzius (CR) cells were observed (Fig. 6A, D). Nectin-1 staining was detected in these cells around the nucleus and in cell processes. Most cells were bipolar, though, some had more than three processes (Fig. 6G). The intensity of the staining was variable, possibly reflecting differences in fixation and the postmortem interval of the

autopsy specimens. In the deeper cortex, nectin-1 immunoreactivity of neurons increased as a function of the distance from the pial surface: nectin-1 positivity
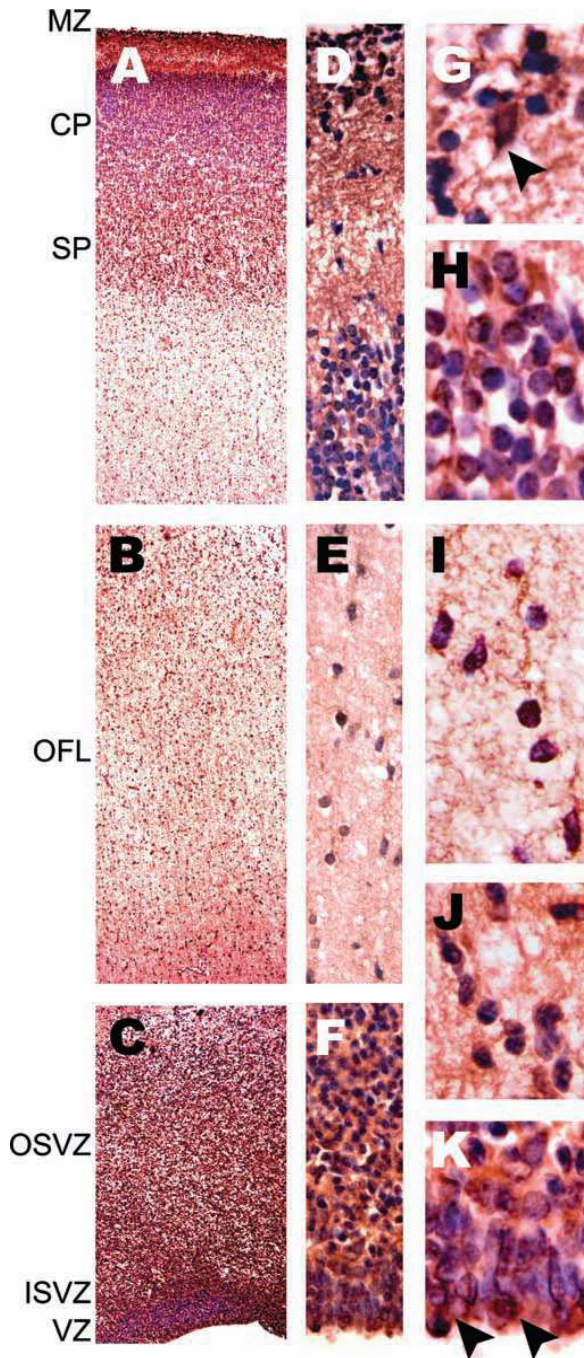

Figure 6 Immunohistochemistry for nectin-1 in fetal human cerebral cortex at 21 GW. Bright field images represent nectin-1-specific staining of the fetal human cerebral cortical wall (**A, B, C**). Cajal–Retzius cells (*arrow*) and subpial granular neurons are strongly nectin-1 positive (**D, G**), whereas subplate neurons are nectin-1 negative surrounded by nectin-1-positive fibers (**H**). As a part of the intermediate zone (**E**), an early appearing outer fibre layer (OFL) forms a nectin-1-positive landmark during development (**I, J**). Nectin-1 is expressed in the cytoplasm and processes of neurons in the deeper layers of the cortex but is essentially absent in more superficial neurons (compare nectin-1-specific staining in **H, I, J**). nectin-1 protein immunosignal was hardly detected in cells characteristic to radial glia like cells in the ventricular zone (VZ) (**K**), but almost absent in the inner subventricular zone (ISVZ) (**C, F**) The outer subventricular zone is histologically similar to the VZ but has a compact radial organization and neurons in this zone express nectin-1 (**J**).
CP, cortical plate; MZ, marginal zone; SP, subplate.

was detected in the cytoplasm and processes of neurons in the deeper layers of the cortex but was essentially absent in more superficial neurons (Fig. 6B, C). Nectin-1 staining was also detected in subcortical migratory neurons (Fig 6E, H, I, and J). In the germinal layer, variable nectin-1 immunoreactivity was detected, ranging from very weak to zero nectin-1 expression in cells close to the ventricular surface to stronger nectin-1 positivity in deeper positioned cells (Fig. 6C, F). The ependymal layer was strongly nectin-1 positive (Fig. 6K).

These findings indicate nectin-1 is widely expressed in the fetal murine and human cortex and suggest that the distribution and intensity of nectin-1 immunoreactivity reflects the order of appearance and degree of maturation of neurons. Furthermore, these findings suggest nectin-1 is strongly expressed in a subset of cells that regulate radial migration of neurons.

Nectin-1 expression in the developing human and murine allocortex

In newborn mice, moderate immunosignal was detected in the entire hippocampus. The immunoreactivity for nectin-1 was demonstrated as small, granule cell–like migrating neurons at stratum radiatum. In addition, moderate to strong immunoreactivity for nectin-1 was also found as traversing postmitotic principal pyramidal cells at stratum oriens. Cell bodies and thin dendrites were labeled in these multipolar immunoreactive cells that were found in the strata oriens and radiatum (Fig. 7A). At postnatal day 7 (P7) and adult hippocampal formation, nectin-1 protein was strongly detected in the hippocampus and the entire dentate gyrus (Fig. 7B, C). The nectin-1 signal was very different from the above described pattern of the P0 stage. There were no phenotypically migrating neurons detectable. In this region, the most intense immunoreactivity for nectin-1 was demonstrated at strata pyramidale and lucidum, along the dendritic trunks of pyramidal cells. In addition to the signals at pyramidal cell layer, faint immunoreactivity for nectin-1 was detected at strata radiatum and oriens.

In the human fetal hippocampal formation, the subgranular layer of the dentate gyrus stained very weakly, suggesting the newly generated neural precursors do not express the entry receptor for HSV, whereas, cells in the
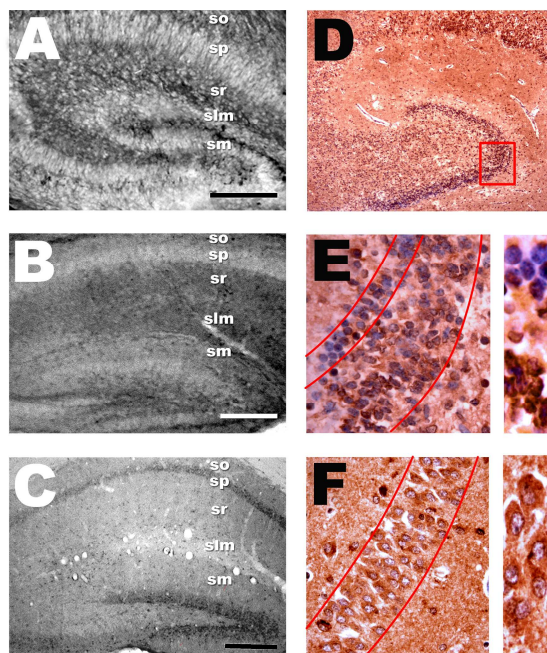

Figure 7
Developmental dynamics of nectin-1–positive fibre tracts and migrating neurons in the hippocampal formation (**A**) On P0, nectin-1–positive bipolar cells are densely packed near the hippocampal fissure in the molecular layer of the dentate gyrus and the hippocampal stratum lacunosum moleculare. (**B**) On P7, the entire hippocampal formation except the pyramidal layer is diffusely stained with anti-nectin-1 antibodies. (**C**) In the adult stage, the nectin-1 immunostaining becomes the direct opposite of the picture seen in earlier stages; the pyramidal layer appears as a strong nectin-1-positive band, whereas in other layers there is only moderate staining. (**D**) Low-power micrograph of the fetal human hippocampal formation stained for nectin-1. The area with red borders indicate the region for higher magnification in **E** and **F**. (**E**) The subgranular layer of the dentate gyrus in human fetal hippocampal formation (**F**)The subgranular layer in the adult human dentate gyrus. Scale bars denote 200μm.
slm = stratum lacunosum moleculare; sm = stratum moleculare; so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum

granular layer were strongly positive. The pyramidal cells of the hippocampus were strongly nectin-1 positive (Fig. 7D-F).

This pattern of protein distribution suggests pyramidal cells turn to radial migration from their tangentially oriented germinal zone, and postmitotic granule cells move across the pyramidal cell layer to their final destination, the stratum oriens.

Our results have shown a specific expression pattern for nectin-1, the main HSV receptor, in the mouse and human brain, and changes in distribution that occur during development. Nectin-1 distribution could explain the marked change in susceptibility to neurotropic HSV infection during brain development. The data suggests nectin-1 expression plays a critical role in determining HSE emergence. Nectin-1 expression renders these cells susceptible to HSV infection and consequent destruction of the cells. The exact molecular mechanism of cellular damage by which HSV induces HSE is poorly understood. It may involve both direct virus-mediated and indirect immune-mediated inflammatory processes. Tissue damage due to oxidative stress can be induced by viral infection. Oxidative stress can also lead to disruption of the blood brain barrier and invasion of immune cells into the CNS. Next, it was determined if HSV-1 infection contributed to HSE development through induction of oxidative stress.

Expression of HSV-1 proteins and HSV-1 replication in P19N neural cultures

To study the impact of oxidative stress and damage on HSV-1 infection, P19N neural cells were used. Neurally differentiated mouse P19 cells (P19N) cells have been widely used as experimental platforms to study neural cells in vitro [66]. First, we needed to determine if P19N cells were susceptible to HSV infection.

To determine whether HSV-1 proteins are expressed in P19N neural cells, P19N cells grown on chamber slides were either inoculated with 1 plaque-forming unit (PFU)/cell of HSV-1 strain KOS or were mock infected with virus free Vero cell extract. Expression of HSV-1 proteins was determined by immunocytochemistry using polyclonal anti-HSV-1 antibody at 24h post infection (p.i.) (Fig. 8). In HSV-1–infected cultures, nearly all cells were immunoreactive for HSV-1 proteins (Fig. 8B). No staining was detected in mock-infected cultures (Fig. 8A) and in virus-infected cultures exposed to secondary antibody only (data not

shown). These observations indicate that HSV-1 proteins are expressed in P19N cells following HSV-1 inoculation.
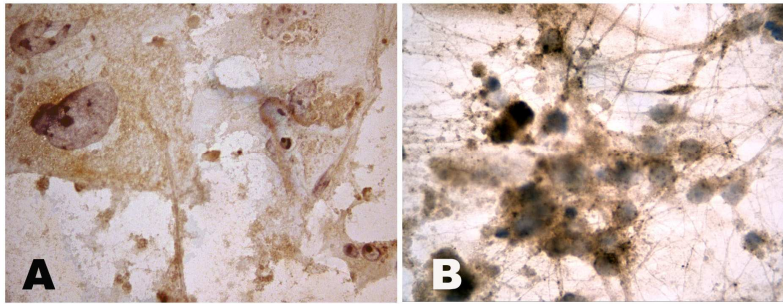

Figure 8 Expression of HSV-1 proteins in P19N neural cultures. P19N cells were either inoculated with 1 PFU/cell of HSV-1 strain KOS or were mock infected with virus free Vero cell extract and the expression of HSV-1 proteins was determined by immunocytochemistry using a polyclonal anti-HSV-1 antibody at 24h p.i. (**A**) Mock infection; (**B**), HSV-1 infection.

To determine whether HSV-1 infects and replicates in P19N neural cultures, P19N cells were inoculated with HSV-1 KOS at a multiplicity of infection (MOI) of 1 PFU/cell, incubated at 37ºC for 1h with occasional gentle agitation, washed twice in sterile phosphate-buffered saline (PBS), and further incubated in fresh medium for 24h. The amount of infectious HSV-1 in the cultures was determined by plaque assay using Vero cells at 1, 6, and 24h p.i. At 1 and 6h p.i., HSV-1 KOS levels in the cultures were significantly lower than the amount of input virus, while at 24 h p.i., KOS titers increased to levels similar to that of input virus (Fig. 9). These observations indicate HSV-1 can replicate in P19N cells, making these cells ,useful for studying the affect of oxidative stress on HSV infection.
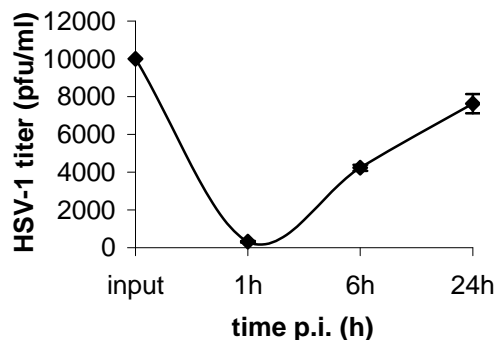

Figure 9
HSV-1 replication in P19N neural cultures following inoculation with 1 PFU/cell of HSV-1 strain KOS as determined by plaque assay. Standard deviation is provided with bars.

Increased generation of ROS in HSV-1–infected neural cultures

To determine whether HSV-1 infection induces ROS in neural cultures, P19N cells were either inoculated with HSV-1 KOS at a MOI of 1 PFU/cell, with Vero cell extract (mock infection), or with sterile PBS. Fluorescent intensity of hydroxyphenyl fluorescein (HPF) was determined at selected times after treatment. Nonfluorescent HPF becomes fluorescent in presence of ROS,

particularly hydroxyl radicals. HSV-1–inoculated cultures demonstrated significantly higher fluorescence than Vero cell extract or PBS-treated cultures at 1, 2, 3, and 24h p.i. (Figure 10). Specifically, HPF fluorescence values in KOS inoculated cultures were 21.5%, 69.8%, 45.7%, and 80% higher than in PBS-inoculated cultures at 1, 2, 3, and 24h, respectively ($p<0.01$ for all time points). Fluorescence values in KOS-inoculated cultures were 38.2%, 32.4%, 35.6%, and 54.3% higher than in Vero cell extract–inoculated (mock-infected) cultures at 1, 2, 3, and 24h, respectively ($p<0.01$ for all time points).
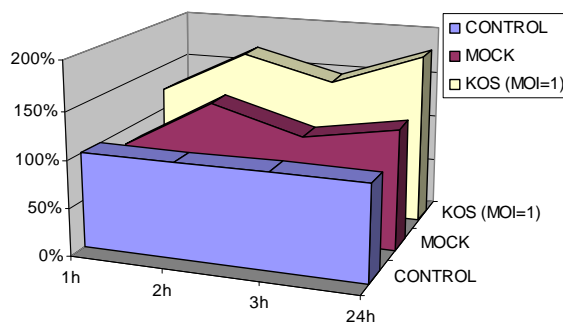

Figure 10
Generation of reactive oxygen radicals (ROS) in P19N neural cells 1, 2, 3, and 24 h following HSV-1 inoculation
HSV-1 KOS, (MOI = 1 PFU/cell), or exposure to PBS or Vero cell extract (mock infection) as detected by hydroxyphenyl fluorescein (HPF) fluorescence. Fluorescence measurements were made using a spectrofluorometer with excitation at 490nm and emission at 515 nm. Results are shown as percent change relative to PBS-treated control cultures.

Inoculation of P19N cultures with heat- or UV-inactivated HSV-1 did not significantly alter ROS levels (Fig. 11). Significantly increased HPF fluorescence, however, was observed following inoculation of P19N cells with HSV-1 strain F and following inoculation of human neural (NT2N) cells with HSV-1 KOS (data not
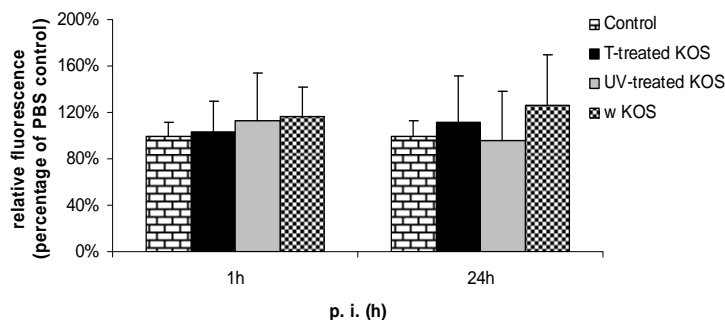

Figure 11
Generation of reactive oxygen radicals (ROS) in P19N neural cells 1 and 24h following HSV-1 inoculation (KOS T-treated. = heat inactivated KOS; KOS UV treated= ultraviolet light-inactivated KOS. MOI = 1 PFU/cell), HSV-1 KOS infection after exposure to heat, UV light or PBS was detected by hydroxyphenyl fluorescein (HPF) fluorescence. Results are shown
as percent change relative to PBS-treated control cultures. The number of examined cultures in each group was 12. Standard deviation is provided with bars.

shown). These findings indicate HSV-1 infection of neural cells is associated with increased ROS generation and suggest increased generation of ROS following HSV- 1 infection requires viral entry and replication.

HSV-1 infection causes lipid peroxidation in neural cell cultures

To determine whether HSV-1 infection causes lipid peroxidation in cultured neural cells, P19N cultures were inoculated with HSV-1 (KOS, MOI = 1 PFU/cell) or exposed to either Vero cell extract (mock infection) or PBS. The combined level of lipid peroxidation products, malondialdehyde and hydroxyalkenals (4-HAE [predominantly HNE]), was then determined in the culture supernatants at 1, 2, 4, 6, and 24h after HSV-1 infection, mock infection, or PBS treatment, using the LPO-586 assay (Bioxytech LPO-586).

HSV-1 infection was associated with increased levels of MDA/HAE in the culture medium at 2 and 4h p.i., but MDA/HAE levels were not different from those detected in mock infected and PBS-treated control cultures at 1, 6, and 24h p.i. (FigA. 12). t 2h following KOS inoculation, MDA/HAE levels were at 358.00 (SD: 20.56) pmol/μg protein, which was significantly higher than MDA/HAE levels at 2h following PBS treatment (296.60 [SD: 2.96] pmol/μg protein; $p<0.01$) or Vero cell extract inoculation (302.00 [SD: 10.10] pmol/μg protein; $p<0.05$). At 4h post-inoculation, MDA/HAE levels were at 486.00 (SD: 57.69) pmol/μg protein, which was significantly higher than MDA/HAE levels at 4h following PBS treatment (302.66 [SD: 9.20] pmol/μg protein; $p<0.01$) and Vero cell extract inoculation
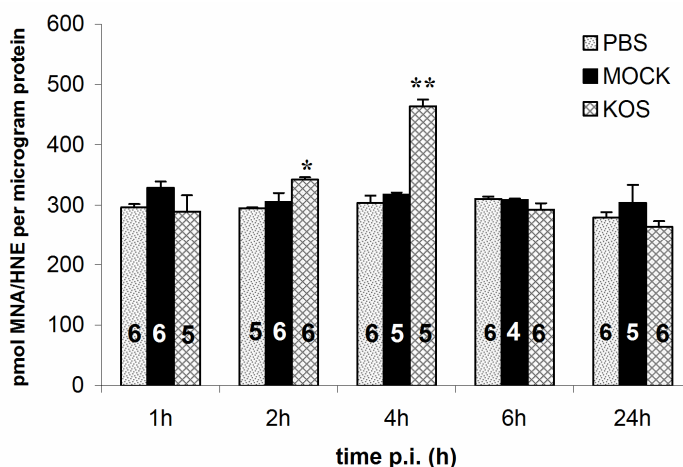

Figure 12
Concentrations of lipid peroxidation products malondialdehyde and hydroxyalkenals (4-HAE, predominantly HNE) in the supernatant of P19N neural cells 1, 2, 4, 6, and 24 h following HSV-1 inoculation Infection with HSV-1 KOS, (MOI = 1 PFU/cell), or exposure to PBS or Vero cell extract (mock infection) as detected by LPO-586 assay (Bioxytech LPO-586). MDA was used as a standard and results are expressed as pmol MDA equivalents/μg protein. Total protein in each sample was determined using Coomassie Plus Protein Assay Kit (Pierce). The number of examined cultures in each group is provided within columns and standard deviation is provided with bars. *,** indicates $p<0.05$, $p<0.01$, respectively, compared to mock infected cells

(310.20 [SD: 8.49] pmol/μg protein; $p<0.01$). These results indicate HSV-1 infection of neural cells induces lipid peroxidation and the release of soluble MDA/4-HAE into the culture medium.

Effect of HNE treatment on HSV-1 replication in various cells lines

Thus far, we have studied how HSV-1 infection leads to oxidative damage. We also studied this relationship from a different aspect and determined how oxidative damage, specifically HNE, and the antioxidant ebselen, affect HSV-1 replication. First, the cytotoxic effect of HNE on Vero, P19, and P19N cell types was determined. Cells were pretreated with HNE at concentrations of 5 or $50\mu M$ or mock treated with ethanol for 0.5 or 1h in low sera containing culture medium at 37℃ in 5% CO_2. Equal distribution of the vehicle (100% ethanol) was used in each sample, including the controls that did not received HNE, at a final concentration of 0.05%. As shown in Figure 13, HNE triggered cell death in a dose dependent manner. Pretreatment with HNE at a high concentration of 50μM caused a dramatic increase in cytotoxicity after both 0.5 and 1h in each cell type. Vero and P19 cells became small and rounded in shape, and P19N cells retracted their cellular protrusion, all of which are common signs of cytotoxicity. Significant toxicity, with approximately 50% cell loss, was detected 1 day following treatment with $50\mu M$ of HNE, a finding similar to previous reports [75]. However, at a lower concentration of 5μM, HNE caused no significant cytotoxicity, which was
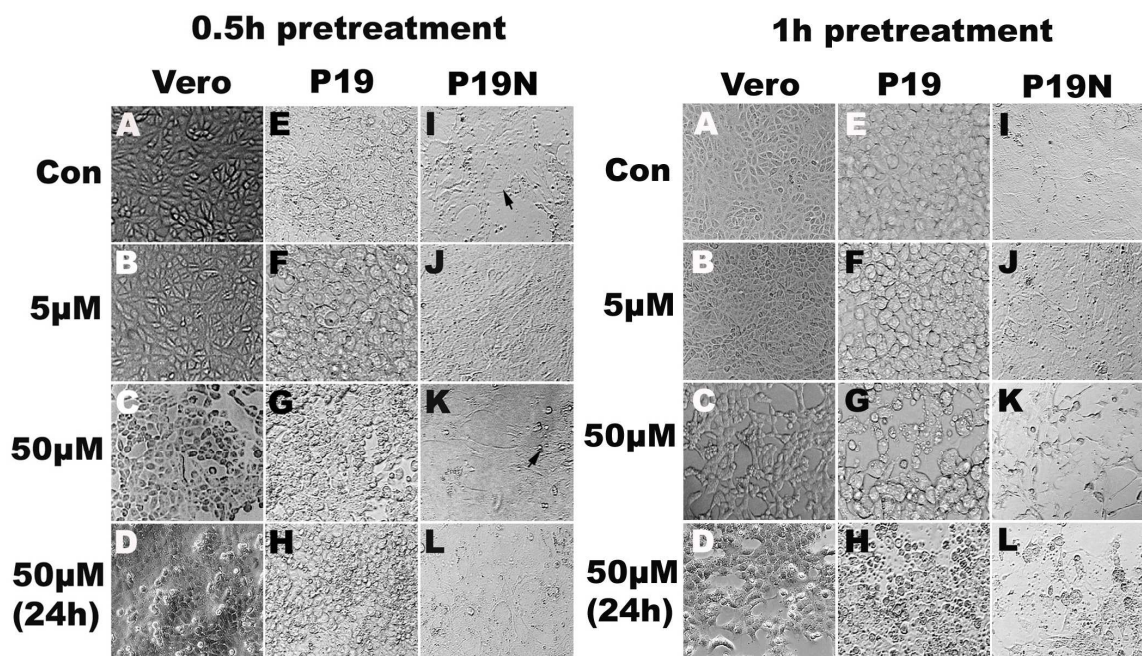

Figure 13
Cytotoxic effect of HNE treatment on Vero, P19 and P19N cells Vero, P19, and P19N cells were treated with HNE at concentrations of 5 or 50μM or mock treated with ethanol for 0.5h or 1h in low sera containing culture medium at 37℃ in 5% CO_2. Equal distribution of the vehicle (100% ethanol) was used in all samples, including the controls which did not get HNE, at a final concentration of 0.05%. I: arrow shows healthy cellular projection of P19N cell. K: arrow points to accumulated death cells on still healthy monolayer.

consistent with previous studies that also indicated these doses correspond to physiologic concentrations *[75, 76].* This showed HNE causes a cytotoxic effect in these cells types in a concentration dependent manner.

To determine if HNE modulates the yield of viral replication Vero, P19, and P19N cells were pretreated with HNE, using the same method described earlier, before inoculation with 0.01 MOI HSV-1 (KOS). Viral replication was compared between HNE treated cells and mock treated cells, which were not exposed to HNE. According to Figure 14, HNE pretreatment at 50μM significantly (p<0.01) decreased viral yields after both 0.5 and 1h in both Vero and P19N cells. In Vero cells, viral yield was reduced to 8.57% (SD: 66.67%) after 0.5h and to 10.53% (SD: 75.00%) after 1h. Similarly in P19N cells, viral yield was reduced to 40.43% (SD: 32.34%) after 0.5h pretreatment with HNE and to 55.96% (SD: 35.40%) after 1h pretreatment. Viral yield in P19 cells was not significantly affected. Altogether, HNE pretreatment at 50μM significantly decreased viral yield in both Vero and P19N cells, though not to the same extent, and this decrease remained around the same levels following 0.5h pretreatment. However, at 5μM, HNE decreased HSV-1 yields only in Vero cells to 34.29% (SD: 25.00%). In case of the P19N cells, HNE at 5μM actually significantly (p<0.05) increased HSV-1 yield to 165.07% (SD: 36.00%) after 1h. There was no significant change in other case.

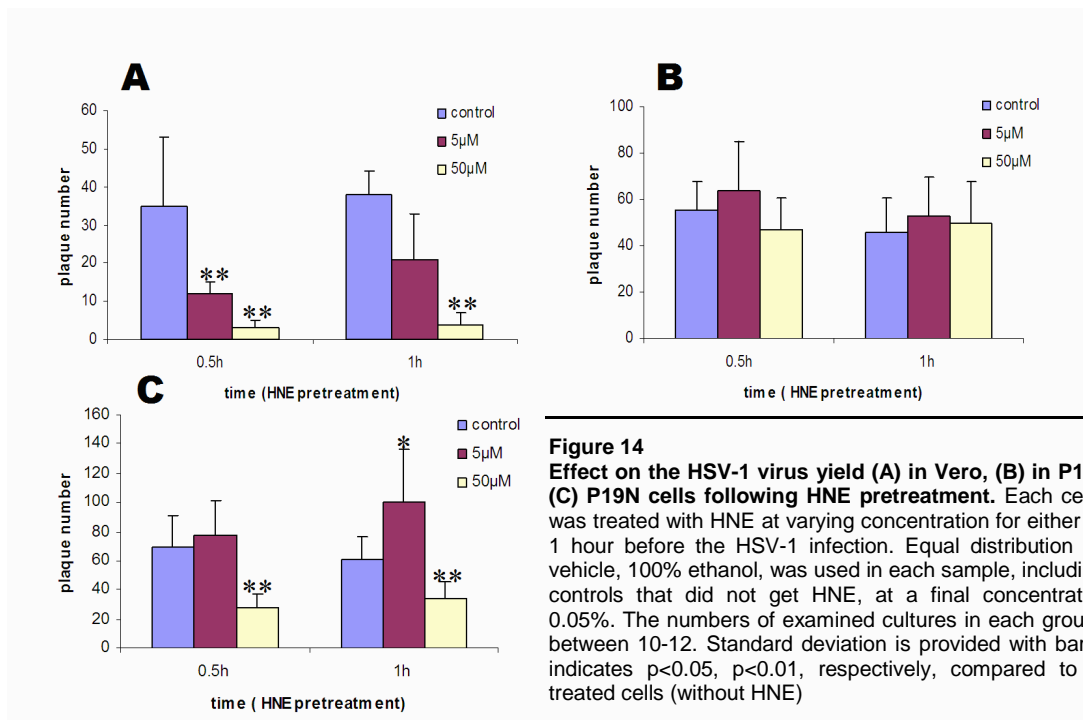Based on our results using neuronal and non-neuronal cell lines, HNE

Figure 14
Effect on the HSV-1 virus yield (A) in Vero, (B) in P19 and (C) P19N cells following HNE pretreatment. Each cell type was treated with HNE at varying concentration for either 0.5 or 1 hour before the HSV-1 infection. Equal distribution of the vehicle, 100% ethanol, was used in each sample, including the controls that did not get HNE, at a final concentration of 0.05%. The numbers of examined cultures in each group was between 10-12. Standard deviation is provided with bars. *,** indicates p<0.05, p<0.01, respectively, compared to mock treated cells (without HNE)

pretreatment can modify the replication cycle of HSV-1 in a cell type and concentration dependent manner. These findings also suggest high concentrations of HNE may reduce HSV-1 replication in P19N cultures by reducing the number of viable cells. Interestingly, the results with P19 and P19N cells show the physiological concentration of HNE alters HSV-1 replication in a cell differentiation dependent manner.

Effect of antioxidant treatment on HSV-1 replication in P19N cells

To determine if antioxidant treatment affected HSV-1 replication in P19N neural cultures, P19N cells were inoculated with HSV-1 KOS at a MOI of 0.01 PFU/cell, incubated at 37ºC for 1h, washed twice in sterile PBS, and further incubated in fresh medium for 24h. Some P19N cultures were treated with 5, 10, or 25μM of the seleno-organic antioxidant compound ebselen for 1h prior to, and for 24h following, virus inoculation. HSV-1 infected cells not treated with ebselen served as a control. Production of infectious HSV-1 virions in culture was determined by plaque assay at 1h, 6h and 24h p.i.

Ebselen treatment reduced HSV-1 yield in a dose dependent fashion (Fig. 15). Relative to untreated KOS infected cultures (100% HSV-1 yield), virus yields were significantly ($p < 0.05$) reduced after 1h p.i. to 50.47% (SD: 12.93%) with 5 μM of ebselen, to 35.02% (SD: 17.50%) with 10μM of ebselen, and to 10.09% (SD: 36.94%) with 25 μM of ebselen. This trend continued after 6h p.i. as reduction of viral yield became further significant ($p < 0.001$). Viral yield decreased to 30.50% (SD: 3.69%) with 5 μM of ebselen, to 23.88% (SD: 1.09%) with 10μM of ebselen, and to 0.95% (SD: 7.03%) with 25 μM of ebselen. Further reduction in viral yield was seen 24h p.i. as well. To determine whether ebselen concentrations used were toxic to P19N cells, the cells were exposed to 5, 10, or 25μM of ebselen in the culture medium without concomitant HSV-1 inoculation. Tthe cultures were observed, using an inverted light microscope, for 26h. No cytotoxic effects were noted following treatment with 5, 10, or 25 μM of ebselen (data not shown). These findings indicate production of infectious HSV-1 in P19N cells is significantly reduced by the antioxidant compound ebselen. This suggests antioxidant treatment could be a possible effective therapeutic agent against HSV

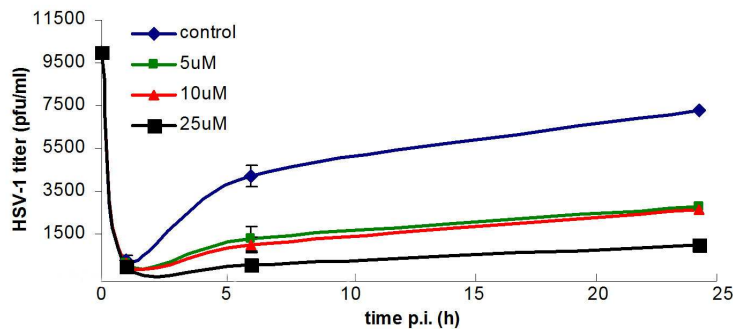infection in the CNS to help prevent the development of HSE and other HSV-induced diseases in the CNS.

Figure 15
Effect of ebselen on HSV-1 replication in P19N neural cells as determined by plaque assay.
P19N cells were inoculated with 0.01 PFU/cell of HSV-1 KOS and a plaque assay was performed 24h p.i. Cultures were treated with 5, 10, or 25μM of ebselen for 1 h prior to and for 24 h following virus inoculation. The number of examined cultures at each experimental group was four and standard deviation is provided with bars.

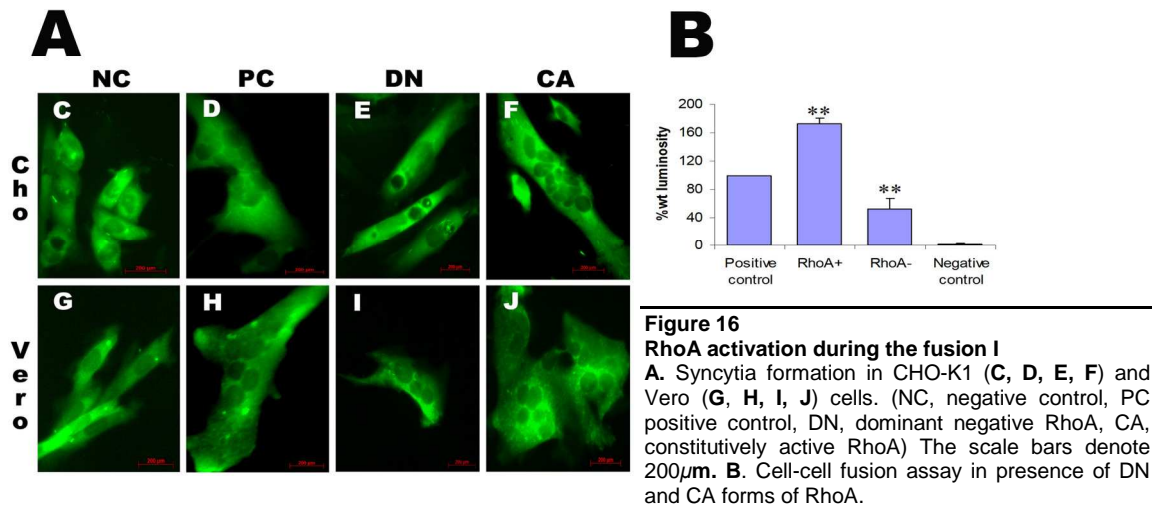## *A role for RhoA signaling in cell- cell fusion and HSE development*

It is believed certain host and viral factors involved in HSV infection play a significant role in HSE pathogenesis, but that role is unknown. Specifically glycoproteins expressed on the viral envelope are critical for HSV entry and cell-cell spread. Our previous work has suggested HSV cell-cell spread is crucial for HSV pathogenesis in the CNS, which could help lead to the development of diseases such as HSE. During this process, HSV glycoproteins are expressed on the surface of the infected cell. This allows the infected cell to bind and fuse with neighboring uninfected cells resulting in multinucleated syncytia formation. This allows the virus to spread through the host while avoiding detection from the immune system. Besides the HSV glycoproteins, cell-cell spread also requires a host cell gD receptor. In neuronal cells, the primary receptor for HSV-1 and -2 is nectin-1. Nectin-1 interacts with HSV gD to mediate entry and cell-cell spread. Though it is known these viral and host factors are required, their exact function during infection, specifically their role in HSE pathogenesis, is unknown.

Studies have shown certain intracellular signaling pathways are activated by interaction between host and viral factors, which could be important for infection. Extensive cytoskeleton rearrangements, which are activated by Rho GTPase signaling pathways, occur during cell-cell fusion. Previous studies have shown Rho GTPases can be activated by nectin-1. *[77-79]*, and also that certain Rho GTPases, specifically RhoA, are activated during HSV-1 entry *[80]*. However, a role for RhoA in HSV cell-cell spread is unknown. It is possible HSV glycoprotein interaction with host cell receptors, specifically with nectin-1, could trigger RhoA
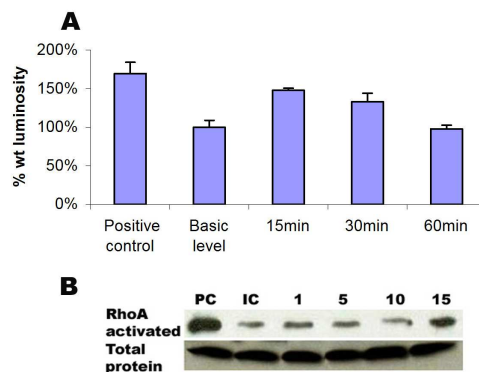
activation during cell-cell spread, and this could be crucial for efficient HSV spread in the CNS and HSE development.

Besides a possible role in HSV spread, RhoA is also activated by ROS, which we have shown is generated during HSV infection. ROS activation of RhoA can disrupt tight junctions and disrupt the blood brain barrier, which leads to invasion of the immune cells into the CNS. Altogether, this suggests RhoA could contribute to more efficient cell-cell spread and the invasion of immune cells into the CNS, all of which could contribute to HSE development.

To study the affect of RhoA on HSV-1 cell-cell spread, a cell-cell fusion model system was used, which can isolate nectin-1 and the four HSV-1 glycoproteins required for cell-cell spread, to investigate their affect on RhoA during cell fusion. Wild type Chinese hamster ovary (CHO-K1) cells were used. A non-neuronal cell line was chosen because RhoA can affect neuronal differentiation *[81]*. CHO-K1 cells are not susceptible to HSV-1 cell-cell spread, but can be made susceptible using transient transfection. One cell population (target cells) was transfected with nectin-1, and the other population (effector cells) was transfected with HSV-1 gB, gD, H and gL. Both populations were also transfected with plasmids expressing either a dominant negative or constitutively active form of RhoA. Both cell populations were mixed in a 1:1 ratio. Fusion was measured using a syncytia assay (Fig. 16A) or β-galactosidase assay (Fig. 16B). In the β-galactosidase assay, CHO-K1 cells expressing the constitutively active form of RhoA showed fusion increased significantly ($p<0.01$) to 172% (SD: 9%) compared to cells expressing vector alone (positive) (Fig 16B). In case of the dominant negative form of RhoA, fusion decreased significantly to 52% (SD: 15%). The syncytia assay showed both the number and size of syncytia increased when constitutively active RhoA was expressed (Fig 16A, panel D). Syncytia size and number decreased when the dominant negative form was expressed (Fig. 16A, panel E). To investigate if the dominant negative or constitutively active form of RhoA had the same effect on a HSV-1 natural susceptible cell line, Vero cells were transfected with dominant negative or constitutively active forms of RhoA, along with GFP, and syncytia formation was analyzed (Fig. 16A, panels G-J). The RhoA plasmids had the same effect on Vero cells as well.

Figure 16
RhoA activation during the fusion I
A. Syncytia formation in CHO-K1 (**C, D, E, F**) and Vero (**G, H, I, J**) cells. (NC, negative control, PC positive control, DN, dominant negative RhoA, CA, constitutively active RhoA) The scale bars denote 200μm. **B**. Cell-cell fusion assay in presence of DN and CA forms of RhoA.

To determine if cell-cell fusion lead to RhoA activation, target and effector cells were transfected as described previously, but without dominant negative or constitutively active forms of RhoA. RhoA activation was measured at selected time points using Western blot analysis with a pull-down assay. It also was determined using G-LISA (Cytoskeleton). Following the manufacture's protocol, G-LISA measured RhoA activation using a luminometer (Tecan) (Fig.17A). RhoA protein expression was measured with Western blot analysis using a pull-down

Figure 17
RhoA activation during fusion II.
A. Results of G-LISA are shown as percent change relative to basic level (100%) of activated RhoA in CHO-K1 cells. Three parallel experiments were performed at each time point. Standard deviation is provided with bars. **B**. Western blot shows activated RhoA expresion up to 15 min. Total protein expression was measured as a controln
IC is the basic level of active RhoA expression at 0 min, For a positive control, cells were treated with GTPγs (PC).

assay with Rhotekin-RBD beads (according to manufacture's protocol) to specifically pull down GTP-bound RhoA to measure active RhoA protein expression (Fig.17B). As shown in Figure 17 the highest level of active RhoA expression was seen at 15min post-mixing in both assays. Active RhoA expression at 15min increased to 148.00% (SD: 2.59%) compared to baseline active RhoA expression. At 30min, active RhoA expression began to decrease and diminished to the basic level by 60min.

To determine ifRhoA activation changed over longer periods of time, target and effector cells were mixed for 2h, 3h, and 5h in addition to the previous time points. Activated RhoA was detected by G-LISA, and cell-cell fusion was verified using a syncytia assay at the first three time points. G-LISA results (Fig.17A and 18A) suggest activation of RhoA i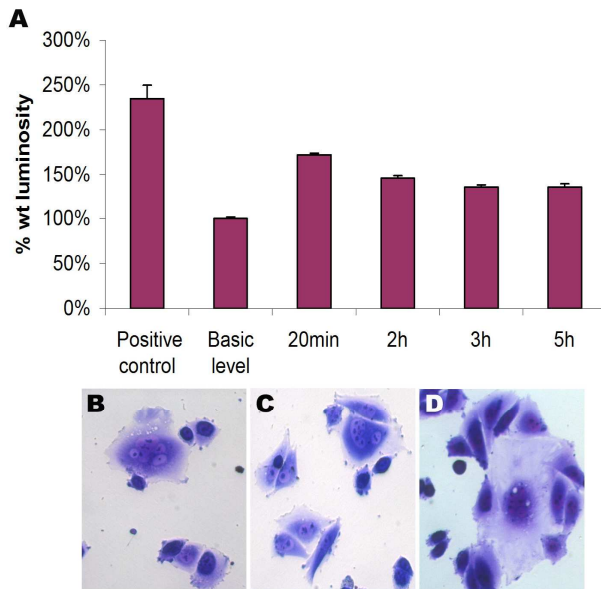s the highest between 15 and 20 min post-mixing. Though RhoA activation declines after this point, higher levels of RhoA activation are seen again at 2h, 3h, and 5h. Active RhoA expression was 145.99% (SD 2.83%) at 2h, 136.15% (SD: 2.38%) at 3h, and 136.18% (SD: 3.43%) at 5h (Fig.18B-D). Following mixing of target and effector cells for the G-LISA assay, small aliquot of cells were placed into a dish and incubated in parallel with the cells in the G-LISA assay for 20 min, 2h, and 3h. After the incubation time was over, cells were fixed and painted with Giemsa to view syncytia formation. Syncytia were visible by 20min (Fig.18B). As the incubation time increased, the number and size of syncytia increased as well (Fig.18 C-D).

Figure 19
RhoA activation during fusion III
A. Results of G-LISA are shown as percent change relative to basic level (100%) of activated RhoA in CHO-K1 cells. Three parallel experiments were performed at each time point. Standard deviation is provided with bars. **B**. Syncytia assay shows the number and size of syncytia formed after 20min (B), 2h (C),and 3h (D)

Our findings show RhoA is activated during cell-cell fusion and its activity has a direct effect on cell-cell fusion and syncytia formation. Peak activation of RhoA occur early, suggesting host cell receptor-HSV glycoprotein interactions are responsible for RhoA activation, which means RhoA may be critical at the start of cell-cell fusion for this process to occur. The fact that activity decreased by 60min but increased again at 2h, 3h, and 5h suggests RhoA may be activated in some separate later signaling event as well, possibly by ROS, or it is activated in a cyclical fashion as new fusion events occur. These results suggest interaction between host cell receptors and viral glycoproteins could be critical for HSV spread in the CNS and thus, could be very important in HSE pathogenesis.

VI.   DISCUSSION

What does the immunolocalization of nectin-1 tell us about the nature of HSE?

HSV-1 is responsible for more than 90% of childhood and adult cases of HSE, while HSV-2 is responsible for most neonatal and occasional adult cases. Neonatal HSE results from disseminated HSV-2 infection in newborns acquired during the genital passage at the time of delivery *[82]*. In newborns with disseminated infection, HSV-2 reaches the CNS by breaking the blood-brain barrier during viremia, and the resulting HSE is characterized by multiple cortical necrotic areas. In contrast, when the disease involves only the CNS of newborn children, neuronal spread is mostly unitemporal with possible bilateral progression of the illness similar to the adult form of HSE *[1, 5]*. Explanations proposed for the site specificity of HSE invoke the routes of access of HSV to the CNS, for instance, viral spread along the olfactory pathways into the nearby temporal and frontal cortices following intranasal inoculation *[2]*. It has also been suggested that infection of the temporal and frontal cortex results from reactivating virus traveling from trigeminal ganglia, the main site of HSV-1 latency *[83, 84]*. Both explanations are plausible, but fail to acknowledge that HSV spread respects the boundaries of an anatomical, developmental and functional neural unit: the limbic system.

It has been postulated that HSE is a consequence of a special affinity of the virus for the limbic system neurons, and the route of entry in the CNS may be of secondary importance *[85]*. Furthermore, the fact that HSE in newborns fails to respect these cytoarchitectonic boundaries emphasizes the spatiotemporal changes in the cell surface protein composition between limbic and nonlimbic neurons. Our results on expression of the HSV receptor nectin-1 at the protein level support this hypothesis. Nectin-1 is expressed in neuronal cells and is the primary receptor for HSV. In the newborn murine brain, the most intense expression of nectin-1 was observed in the cerebral cortices and in the interconnecting structures of the hemispheres (the corpus callosum, commissura hippocampi, and anterior commissure). After day P7, another pattern emerged: the nectin-1 expression selectively increased in limbic regions and concomitantly decreased in association areas. In the adult brain and in the neonatal brain,

spatial expression of nectin-1 correlated with areas affected by HSE in the limbic system. Moreover, developmental regulation and loss of nectin-1 expression in the corpus callosum correlated with limited unilateral HSV spread in adult HSE. We propose that this developmental change in the virus receptor distribution as an explanation to the marked change in susceptibility to neurotropic HSV infection during brain development.

What does the immunolocalization of nectin-1 tell us about the HSV-1 susceptibility of the developing brain?

Clear and consistent changes in the neuroanatomical distribution of nectin-1 were observed with age. Our results detailed the changes occurring in neural systems where neuronal maturation patterns have been extensively studied.

It has been known for a long time that in rodents, there is a marked change in susceptibility to neurotropic virus infection during the first few postnatal days *[86]*. Under evolutionally pressure of natural selection, neurotropic viruses target the transcriptionally/translationally active subset of neuronal precursors. We demonstrate the existence and change in distribution of a special population of immature neural migratory cells with the nectin-1 marker. To understand the syndromes that are suggested to be the result of HSV infection of the CNS, it is very important to take into account that the main targets of HSV are migrating cells. We have shown here that nectin-1, the major entry receptor of HSV, is widely expressed in the immature human and murine brain. Nectin-1 is widely and strongly expressed in the leptomeninges and in ependymal cells and is expressed in a maturation-specific manner in neurons. Neurons in the deeper layers of the neocortex, in the hippocampus, and in the ventricular zone are nectin-1 positive. Interestingly, the undifferentiated cells in the ventricular zone, and neurons in the outermost areas of the fetal neocortex were nectin-1 negative. Importantly, we detected strong nectin-1 expression in a subset of cells that regulate migration of neurons, including CR neurons and radial glia cells.

Neonatal HSE is characterized by widespread cortical necrosis and is typically associated with a more severe and devastating process than in the adult brain. Similarly to humans, neonatal mice are much more sensitive to HSV infection than adult mice *[87]*. An increased susceptibility of the immature brain to

HSV was also observed in the *ex vivo* organotypic brain slice infection system *[88]*. HSV-1 infection of brain-tissue slices was mostly localized at specific regions: the periphery of the brain, consistent with leptomeningeal and cortical cells, and in the hippocampus of neonatal mouse and neonate rat brain. Neonate brain tissues were much more permissive to HSV-1 infection than adult mouse brain tissues *[88].* Therefore, this *ex vivo* model, lacking a systemic immune system, excludes the possibility that this observation is related to immune competence and favors the possibility that an increased number of susceptible cells in the neonatal brain tissue are responsible for the augmented neuronal infection. These support our earlier findings that the susceptibility of immature neurons to HSV may be related to an increased expression of neuronal surface molecules acting as viral receptors.

Widespread expression of nectin-1 in brain regions known to have increased susceptibility to HSV infection in the immature brain suggests nectin-1 expression plays a critically important role in regulating HSV pathogenesis in the fetal and neonatal brain and HSE development. Particularly important could be our finding that nectin-1 is expressed in cells important for the guidance of migrating neurons. Nectin-1 expression in these cells may render these cells susceptible to HSV infection and consequent viral destruction of these cells may be critically important for neuronal migration problems associated with HSV infections.

What does the immunolocalization of nectin-1 tell us about the structural development of the brain?

In the postnatal period, a wave of secondary neurogenesis produces huge numbers of neurons destined for the cerebral cortex, the hippocampal formation, and the olfactory bulb *[63]*. In the cerebral cortex and the hippocampal formation, postmitotic neurons migrate radially away from the germinal zones lining the ventricles. Cortical neurons are generated within the proliferative layers adjacent to the cerebral ventricular system and follow a radial gradient of migration and positioning, which determines the characteristic layering and pattern of neural connections in the adult cerebral cortices. Thus, directional migration of postmitotic neuroblasts towards their final positions and regulation of the radial-guiding cell phenotype (e.g., Cajal-Retzius, radial glia) subserving cortical

migration are central issues in corticogenesis *[89]*. Malformations attributed directly or indirectly to infection of the radial glial scaffolding have been observed in both primate and nonprimate mammals. One scenario may be that various agents that interfere with neuron-glia interaction, and thus indirectly impair neuronal proliferation and their path finding or motility, can, even with very little or no injury to neurons, prevent their normal placement.

Our results demonstrate changes in nectin-1 expression occur in neural systems, where neuronal maturation patterns have been extensively studied. Neuronal stem cells produce neuroblasts that migrate from the subventricular zone along a discrete pathway to the cerebral cortex where they form mature neurons. Another neurogenic region is the subgranular layer of the hippocampal dentate gyrus, where neurons migrate only a short distance and differentiate into hippocampal granule cells. Distribution of nectin-1 coincided with the HSV-susceptible cell population and with localization of guiding cells that regulate radial migration of neurons in cortical lamination and also in migrating neural precursor cells in the developing brain.

We demonstrated in all areas a specific maturation and migration-dependent change in nectin-1 expression in neurons of the immature brain, which has led us to develop the following model of radial migration: nectin-1 is not expressed in the
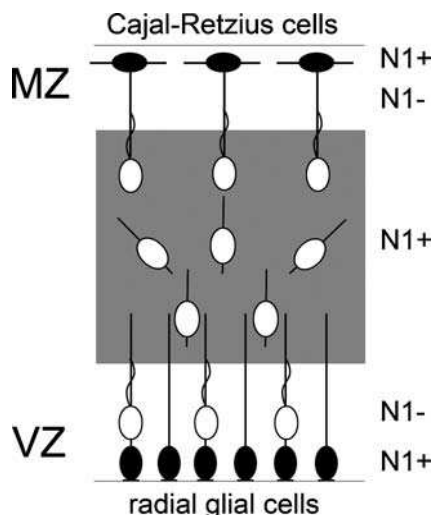

Figure 19 A model on nectin-1–sensitive neuronal migration in the mammalian neocortex. In the ventricular zone, immature neuroblast are attached to nectin-1-positive radial glia and advance towards the CP via glia-guided locomotion. In the next step of their migration, they detach from the glia fibers and migrate tangentially under the influence of nectin-1. Nectin-1 expressing Cajal-Retzius cells send radial processes from the apical surface and these guiding processes are needed to lead the migratory cells to their final position. Once migrating neurons reach their intended position, nectin-1 is expressed again to augment synaptogenesis

most immature neuroblast but becomes expressed in immature neurons that are about to begin migration. Nectin-1-expressing guiding cells send radial processes, and these guiding processes are needed to lead migratory cells. Once migrating neurons reach their intended position, nectin-1 is again expressed to augment synaptogenesis (Fig. 19).

In the cerebral cortex and the hippocampal formation, postmitotic neurons migrate radially away from the germinal zones lining the ventricles, whereas in the olfactory bulb tangential chain-migration occurs *[63, 64]* Although well-documented *[63-65]*, the interaction of the radial and tangential migratory systems during brain development is still unclear. Our study suggests that nectin-1 is involved in both migratory processes. The distribution of nectin-1 in the developing brain suggests an important role of the intermediate zone in organizing the tangentoradial transition. A similar inference was drawn by Okabe et al *[90]* from an analysis of nectin-1 function in a flat-mounted open-book hindbrain model. That study demonstrated that the interaction between nectins at the contacts between commissural axons and floor plate cells was required for the longitudinal turns of the commissural axons. The present analysis indicates that, during the middle and late stages of neuronal migration to the superficial and middle layers of the neocortical plate and hippocampus, nectin-1–positive fiber tracts guide postmitotic young neurons across the intermediate zones to their final destinations. In the same way nectin-1 positive fibers guide commissural fiber tracts migrating through the midline in the early stages of perinatal brain development.

These findings indicate that nectin-1 is widely expressed in the fetal murine and human cortex and suggest that the distribution and intensity of the nectin-1 immunoreactivity reflects the order of appearance and the degree of maturation of neurons. Furthermore, these findings suggest nectin-1 is strongly expressed in a subset of cells that regulate radial migration of neurons

We have demonstrated the nectin-1 expression pattern could be crucial for HSV pathogenesis in the brain and for determining the emergence of HSE. Nectin-1 expression renders these cells susceptible to HSV infection, allowing for the destruction of these cells and the development of HSE. Though, the molecular mechanism as to how HSV induces HSE is poorly understood. It appears tissue damage caused by HSV-1-induced oxidative stress could be critical for the development of HSE.

Does oxidative stress and damage have a considerable role in the manifestation of HSE?

We saw how primary infection of HSV-1 can lead to typical malformations of the HSE-brain in neonates due to failed neuronal migration. But only one in three cases of HSE is due to primary HSV infection. The remaining two-third occurs in patients with serologic evidence of preexisting HSV infection and is due to either reactivation of a latent peripheral infection in the olfactory bulb or trigeminal ganglion, or reactivation of a latent infection in the brain itself. Molecular mechanisms by HSV infections cause nervous system disease, like HSE, are diverse and poorly understood. Previous studies have reported that neurons and non neural cells are quickly overwhelmed by a lytic and hemorrhagic process during this life threatening disease, this escorted by infiltration of brain by acute and chronic inflammatory cells and tissue necrosis and apoptosis of neuronal and non neuronal cells. *[30, 91, 92]*. The exact mechanism of cellular damage is unclear, but it may involve both direct virus-mediated and indirect immune-mediated inflammatory processes. It is likely that the host immune and inflammatory responses play an important role in HSE pathogenesis.

Increased generation of ROS and RNS is a feature of many viral infections *[93]*. Injury produced by ROS and RNS is an important common pathway of tissue damage. Tissue damage due to oxidative stress can be induced by viral infection involving HSV *[94]*. Differentiated monocytes, macrophages, murine neurblastoma, and coronary artery smooth muscle cells has been already shown that HSV-1 infection induce increased production of ROS and RNS *[95, 96]*. The mechanism(s) involved in HSV-1-induced directly oxidative stress in neural cells (neurons and glias) remains unknown at this point. The observation that HSV-1 infection leads to increased ROS levels in neural cells is novel and extends our existing knowledge related to the capacity of viruses to directly cause oxidative stress in cells. We have previously mentioned that HSE development is most likely due to both viral-mediated and immune-mediated inflammatory responses. The generation of ROS and development of oxidative stress and oxidative damage could lead to both. Not only can HSV infection induce oxidative damage and contribute to HSE development, but ROS generated by HSV infection can also disrupt tight junctions which can break down the blood brain barrier, allowing

immune cells to invade the CNS. These immune cells can cause further tissue damage and further contribute to HSE.

Our study demonstrates that HSV-1 replicates in P19N neural cells and that HSV-1 infection leads to increased ROS levels in these cells. HSV-1 infection leads to increased ROS levels as early as 1 hour p.i. and ROS levels remain elevated in infected cells until 24 hours p.i. We have also provided evidence suggesting that the increased generation of ROS following HSV-1 infection of neural cells requires viral entry and replication because infection with heat- or UV-inactivated HSV-1 did not induce increased ROS levels. Detection of increased ROS levels in neural cultures as early as one hour following HSV-1 inoculation suggests that newly expressed viral immediate early or early gene products are sufficient for HSV-1-induced oxidative stress. However, some studies have shown immediate early gene expression does not start until 2 hours after entry, so we must consider the possibility that certain viral-host cell interactions, possibly with nectin-1, during entry or post-entry steps could contribute to ROS generation.

Steady state levels of oxidative tissue damage represent a balance between rates of damage caused by pro-oxidant stimuli and rates of antioxidant and tissue repair mechanisms that decrease ROS/RNS levels and remove oxidatively damaged molecules [41, 97]. Components of the antioxidant defense system include enzymes, like superoxide dismutases, and glutathione peroxidase, such as ebselen [98, 99], and antioxidants, like vitamins E and C. We have found that HSV-1 replication in P19N cells is inhibited by the treatment of cultures with the antioxidant compound ebselen. Our study shows HSV-1 replication in P19N cells is inhibited by ebselen in dose dependent fashion, suggesting HSV-1-induced oxidative stress is required for efficient viral replication in neural cells. These observations extend previous studies indicating antioxidants can inhibit HSV-1 replication *in vitro [100, 101]*. It is also noteworthy that antioxidant therapy has been reported to decrease the severity of HSV-1 ocular infection in mice *[102]*, and raises the importance of antioxidant therapy in clinical practice.

Free radicals can cause lipid peroxidation, an autocatalytic process that damages lipid-containing structures and yields soluble, highly reactive by-products, primarily 4-hidroxy-2-nonenal (HNE) and malondialdehhyde (MDA). HNE and MDA are electrophilic species that covalently modify and damage cellular macromolecules. HNE induces cell death and DNA fragmentation in a

dose dependent manner [103-106] Studies using a murine HSV-1 neuropathogenesis model show HSE is associated with neuronal and non neuronal HNE and 8-hydroxyguanosine (8-OHG) specific immunoreactivity in acute HSV-1 infected areas of the brain, indicating oxidative damage to proteins and nucleic acids [94]. Furthermore, levels of F_2-isoprostanes and F_4-neuroprostanes are markedly elevated during acute HSV-1 encephalitis, indicating damage of arachidonic acid and docosahexaenoic acid, respectively [107, 108]. Levels of these lipid peroxidation compounds become modest, but are still consistently detectable during latent HSV-1 infection [94],[109].

We have demonstrated that HSV-1 infection induces increased levels of soluble lipid peroxidation by-products MDA/HAE (predominantly HNE) in the culture medium of neural cells at the early stages of the infection. This observation indicates that HSV-1 infection can directly cause oxidative damage in neural cells *in vitro* and suggest that HSV-1 may directly contribute to HSE development. It is interesting that although HSV-1 induced persistently elevated ROS (oxidative stress) in neural cells in our studies, lipid peroxidation (oxidative damage) was observed only during the early stages of the infection. The mechanism and relevance of this observation is unclear at this point. However, it is possible that neural cells can mount an effective antioxidant defense during the later stages of HSV-1 infection. In that respect it is noteworthy that increased antioxidant enzyme activity following HSV-1 infection has been reported in non-neural tissue culture systems [110]. It should also be noted that the possibility of the expression of an HSV-1 gene(s) with antioxidant function at the later stages of the viral replication cycle in P19N cells can not be excluded.

The fact that HSV-1 infection of neural cells is associated with an increased release of highly bioactive lipid peroxidation by-products MDA and HNE *in vitro* raises the possibility of a novel mechanism by which HSV-1 may affect the physiology and may damage uninfected bystander cells in the nervous system *in vivo*. At physiologic concentrations, HNE and MDA are potent regulators of cell growth and differentiation, affecting both cellular transcription and cell cycle progression [43, 44, 111, 112]. Also HNE has ability to increase HSV-1 permissiveness in neuronal cultures at physiological concentration. Latent HSV-1 infection of murine nervous system is associated with persistent, chronic inflammation and levels of lipid peroxidation by-product such as HNE, 8-OHG, F_2-

isoprostanes, and F_4-neuroprostanes, are still consistently detectable in HSV-1 latent infected areas of the brain *[94, 109]*. Our finding could be very important because during reactivation, the already present HNE could accelerate HSV-1 replication and help overcome the antioxidant defense system to contribute to developing HSE. In neuronal cell cultures, HNE has been reported to activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, inhibit NF-kB activity, and induce apoptosis in a dose dependent manner *[44, 103, 105, 106]*. NF-kB activity and other cell activation signals, as well as, the differentiation state of cells are known to be affected by HSV-1 infection and are known to participate in the regulation of the HSV-1 replication cycle *[113]*. Thus, HNE and MDA released from HSV-1 infected cells may not only induce cell death in uninfected bystander cells but may also modulate their permissiveness to HSV-1 infection in the nervous system. Our findings indicating that HNE can modulate HSV-1 replication in P19N cells provide strong support for this latter possibility, the mechanism by which oxidative stress would promote HSV-1 replication in neural cells is not evident from our findings though. In summary, our observations may lead to a better understanding of the pathogenesis of HSV-1 encephalitis including the mechanisms of tissue injury and the regulation of viral replication in neural tissues.

Could the Rho GTPase RhoA be a link between oxidative stress and HSV infection?

All CNS pathogens, including HSV, use the host intercellular structure and host intracellular signalization cascades to enter the host neuron and replicate. The basis for these processes is molecular mimicry.

Reorganization of the actin cytoskeleton plays a crucial role in many cellular functions such as cell morphology reorganization, cell motility, cell adhesion, cell migration, and cytokinesis. Small GTPases act as central players or linkers between these different signaling cascades, thus they have an important role in all of these processes *[114]*. Cell surface or intracellular perturbation, such as virus infection, uses these signal transduction pathways to alter cytoskeleton structure, induce vesicle transport, and alter transcription factor activity *[115]*.

Several works have discussed how essential the host cytoskeleton system is for the effective infection of a large variety of viruses, including retroviruses [116], influenza [117], adenovirus [118], vaccinia [119], pseudorabies virus [120], Kaposi's Sarcoma associated herpes virus [121]. A number of live cell studies suggest that temporal and spatial separation of virus attachment and internalization through filopodia and lamellopodia formation frequently occurs during the establishment of infection [122]. Also, many viruses are transported on microtubules from the plasma membrane toward the nucleus during the establishment of infection, and later from their replication site toward the periphery as they leave the host cell [123, 124]. Cell-cell spread is one of the primary ways HSV spreads to the CNS and through the CNS. High nectin-1 expression in the CNS allows for efficient cell-cell spread. Extensive actin cytoskeleton reorganization occurs during cell-cell spread, and these rearrangements usually require activation of Rho GTPases. Nectin-1 trans-interactions can activate Rho GTPases [77, 79]. It is possible nectin-1 interaction with HSV-1 gD mimics this and activate Rho GTPases..

Previous studies have shown that specific small Rho GTPases are activated during HSV-1 entry, and their activity allows for more efficient HSV entry. Specifically, activation of the Rho GTPase RhoA has shown to be very important for more efficient HSV entry [80]. RhoA activates multiple effectors, such as Rho kinase (ROCK), and functions in multiple signaling pathways. One of these pathways seems to facilitate HSV entry. How RhoA is activated and what its role in HSV cell-cell spread is currently unknown.

RhoA signaling could be critical in not only more efficient spread. Previous studies have shown Rho signaling can disrupt blood brain barrier [50] by interfering with tight junctions. Both nectin-1 [77, 79] , and ROS formed by HSV infection can activate Rho signaling. So not only could Rho signaling be important for HSV spread throughout the CNS, but it could also disrupt the blood barrier allowing immune cells to enter the CNS. Both processes could lead to a significant more amount of tissue damage, which could contribute extensively to the development of HSE. Thus, study RhoA during cell-cell spread could be very important in determining how HSE develops.

To determine if RhoA was activated during HSV-1 cell-cell spread, we used a model system, allowing us to specifically investigate the affect of nectin-1 and the

HSV glycoproteins on RhoA signaling during cell-cell fusion in CHO-K1 cells. Cells infected with HSV form multinucleated cells, or syncytia, as a result of viral glycoprotein expression on the surface of infected cells [125]. It is also known RhoA signaling can be required for viral filaments and syncytia formation, such as in RSV [126, 127]. Furthermore, overexpression of citron, another effector of RhoA, results in the formation of multinucleated cells [128]. In our syncytia assay, both cell populations were transfected with plasmids expressing either a dominant negative or constitutively active form of RhoA. Both the number and size of syncytia increased when the constitutively active form was expressed, and decreased when the dominant negative form of RhoA was expressed. Our findings suggest RhoA activity has a direct effect on cell-cell fusion and syncytia formation, which can act as virus factories because they can evade the host defense mechanism more effectively. Thus, RhoA could be critical for efficient HSV-1 cell-cell spread in the CNS.

In addition, western blot analysis and the G-LISA assay showed RhoA was activated during cell-cell fusion, with higher levels of cell-cell fusion associated with significantly higher levels of activated RhoA from 15 min post-mixing to the next few hours. Maximum activation occurring between 15 and 20 min corresponded with the findings of Clement et al [80]. The fact that activity decreased by 60min, but it was significantly high again at 2h, 3h, and 5h, suggests that Rho signaling may take part in some late signaling events as well. It is could be possible RhoA is activated during the initial events of cell-cell fusion and only activated later on after the cell contents have mixed and a new fusion event can occur. It is also possible nectin-1 can initially activate RhoA during the earlier events of fusion, and later on RhoA is again activated, but this time by ROS that has been generated due to HSV infection.

Cell-to-cell fusion mediated by some viral envelope proteins involves the cellular actin cytoskeleton .[129]. The early peak of activation raises the possibility that some early RhoA mediated event, such as the formation of focal adhesions from actin stress fibers [130] and regulation of cell-to-cell adhesion [55, 131, 132], is important for successful HSV infection. RhoA is responsible for forming actin stress fibers: bundles of actin filaments that traverse the cell and are linked to the extracellular matrix through focal adhesion plaques [133]. Interestingly, nectin-afadin system associates directly with actin filaments, and it is already known that

the interaction of nectin-1 with afadin increases the efficiency of intercellular spreading of HSV-1 [10]. It suggests the importance of role of the actin cytoskeleton in cell-cell spreading of HSV. It is also reported, that nectin-afadin system associates directly with the peripheral actin binding membrane proteins of tight junction (ZO-1, 2, 3), thus nectin-1 may play a role in the organization of tight junctions [20]. So it is possible that besides RhoA activation, HSV-1 interactions with nectin-1 could have a direct effect of the rearrangement of tight junctions through nectin-afadin system.

It has been shown that there is infiltration of the HSV infected brain by acute and chronic inflammatory cells [30, 91, 92]. It is known that blood monocytes migrate early to foci of HSV infection [134]. ROS are produced within minutes upon the interaction of monocytes with brain endothelial cells. In addition, administration of exogenous ROS to brain endothelial cells induces cytoskeletal rearrangement [135]. It has been revealed that monocytes can activate the Rho/Rho kinase signaling pathway, which leads to loss of tight junction integrity due to phosphorylation of tight junction proteins that contribute to impairment of blood brain barrier and monocyte migration during HIV encephalitis [136]. Monocytes most likely activate RhoA/Rho kinase through the PKCα pathway [137]. Furthermore, antioxidants are able to reduce RhoA activity [138] and prevent transendothelial migration of monocytes [135]. Lymphocyte migration through the blood brain barrier also involves a Rho-dependent pathway mediated by ICAM-1 [139]. Lymphocyte binding to another adhesion molecule, VCAM-1, activates endothelial cell NADPH oxidase. This is required for VCAM-1-dependent lymphocyte migration across the blood brain barrier [140] in experimental allergic encephalomyelitis. VCAM-1 lymphocyte interaction activates NADPH oxidase, which induces transient PKCα activation [140]. ROS can stimulate activation of PKCα through oxidation of its regulatory domain [141]. These studies reinforce our suggestion that ROS generated by HSV infection, and possibly nectin-1 itself, activate the RhoA signaling pathway via PKCα, which leads to blood brain barrier impairment and inflammatory cell infiltration during HSV infection. ROS can then be generated in the inflammatory cells themselves and cause further damage. This chain reaction could lead to significant tissue damage and contribute extensively to HSE emergence.

Interestingly, RhoA also has an anti-apoptotic function *[142]*. It has been shown that PKD activation has a critical role in oxidative stress-induced epithelial cell injury, where PKD activity was regulated by PKC-δ downstream of the Rho/ROK pathway *[143]*. The virus needs the host to survive so HSV could be initiating Rho signaling to aid in host cell survival in the CNS.

RhoA's function in multiple pathways makes it very complex and further work must be done to better understand its relationship with HSV and its contribution to HSE. Further work must also be done with other Rho GTPases (eg. rac-1) to determine their relationship with HSE. Nonetheless, it appears that RhoA could be crucial in both direct virus-mediated (HSV cell-cell spread) a immune-mediated inflammatory processes (inflammatory cell invasion supported by Rho signaling) that lead to HSE development.

VII.   SUMMARY

HSV-1 is responsible for most cases of HSE in both children and adults, while HSV-2 primarily induces HSE in most neonatal cases and occasional adult cases. Neonatal HSE can involve any, and often multiple, parts of the brain, in contrast with the typical temporal lobe predilection and mostly unitemporal neuronal virus spread seen with HSE that has onset beyond the neonatal period *[1, 5]*. Nectin-1 expression selectively increased in limbic regions and concomitantly decreased in association areas during postnatal brain development. Developmental regulation and loss of nectin-1 expression in the corpus callosum correlated with limited unilateral HSV spread in adult HSE. We propose this developmental change in virus receptor distribution as an explanation to the marked change in susceptibility to neurotropic HSV infection during brain development.

Pathological manifestations of intrauterine HSE are cortical atrophy, hydrocephalus, microcephaly, periventricular leukomalacia, and microphthalmia *[6]*. These brain malformations are suggested to be the result of direct viral destruction of the CNS. Recently, it was revealed nectin malfunction in animal models showed similar disturbances in eye and periventricular brain development *[144, 145]*. These findings, along with our results, suggest the intrauterine ablation of preplate cells due to HSV infection dramatically alters the cellular lamination and connectivity of the cortical plate. In parallel to the cell death within the proliferative zones, HSV envelope protein gD influences nectin-1 function, leading to early disruption of the radial glial framework and subplate structure in the developing cortex and to an impaired radial migration of neurons into the cortical plate from the ventricular zone. After birth, a cortical lesion develops, which becomes exacerbated with the secondary onset of hydrocephaly in the postnatal migratory process. The result underscores the critical importance of the nectin-1– positive proliferative zone in cortex formation, mediated through its guidance of the formation of radial glial scaffolding, subsequent neuronal migration into the incipient cortical plate, and final arrangement of its vertical organization and cellular connectivity.

Our results demonstrated changes in nectin-1 expression occurred in neural systems where neuronal maturation patterns have been extensively studied. In the cerebral cortex and the hippocampal formation, neuronal stem cells produced

neuroblasts that migrated radially from the germinal zone along a discrete pathway to the cortex where they formed mature neurons. We demonstrated in all areas a specific maturation and migration-dependent change in nectin-1 expression in neurons of the immature brain, which led us to develop a model of nectin-1–sensitive radial migration. In the olfactory bulb where tangential chain-migration occurs, we identified two similar, but fundamentally different, migratory systems based on nectin-1 expression: the rostral (RMS) and the callosal migratory stream (CMS). Although well-documented *[63-65]*, interaction of the radial and tangential migratory systems during brain development is still unclear. Our study suggests nectin-1 is involved in both migratory processes.

Nectin-1 distribution coincided with the HSV-susceptible cell population and with localization of guiding cells that regulate radial migration of neurons in cortical lamination and in migrating neural precursor cells in the developing brain. These findings suggest intrauterine ablation of preplate cells due to HSV infection dramatically alters cellular lamination and connectivity of the cortical plate. Besides nectin-1 distribution, it is possible the type and distribution of other potential receptors may modify the pathological picture of the disease, depending on HSV type.

Here we demonstrated nectin-1 expression could play a critically important role in determining the emergence of HSE. Nectin-1 expression in these cells renders them susceptible to HSV infection and consequent destruction of these cells. Thus far the exact molecular mechanism by which HSV infection induces HSE is poorly understood, but our results suggest that generation of ROS and activation of the RhoA signaling pathway by ROS during cell-cell spread may be involved in both direct virus-mediated and indirect immune-mediated inflammatory processes that lead to the development of HSE.

Increased generation of ROS and reactive nitrogen species (RNS) is a feature of many viral infections that commonly leads to tissue damage due to oxidative damage. It was previously unknown if HSV-1 could directly induce oxidative stress and oxidative damage in neural cells (neurons and glias). P19N cell susceptibility to HSV infection was shown by immunohistochemical observation. The results showed HSV-1 proteins were expressed in P19N cells following HSV-1 inoculation, and plaque assays showed HSV-1 was able to replicate in P19N cells as well. Thus, these cells were useful for studying the

affect of oxidative stress on HSV infection. The results showed HSV-1 infection of neural cells was associated with increased ROS generation and suggested increased ROS generation following HSV-1 infection required viral entry and replication. Detection of increased ROS levels in neural cultures as early as one hour following HSV-1 inoculation proposes newly expressed viral immediate early or early gene products are sufficient for HSV-1-induced oxidative stress, or possibly entry or post-entry events could cause ROS generation. In addition, ROS are involved in the disruption of tight junctions which can lead to migration of inflammatory cells into the CNS, which cause even further tissue damage.

Homeostasis must be maintained between rates of damage caused by pro-oxidant stimuli and rates of antioxidant and tissue repair mechanism that decrease ROS levels and remove oxidatively damaged molecules [41, 97]. ROS generation by HSV infection disrupts this balance. Components of the antioxidant defense system include enzymes, like glutathione peroxidases such as ebselen [98, 99]. Our study showed that HSV-1 replication in P19N cells was inhibited by ebselen in a dose dependent fashion, suggesting HSV-1-induced oxidative stress is required for efficient viral replication in neural cells. These observations extend previous studies indicating that antioxidants can inhibit HSV-1 replication, and it raises the importance of antioxidant therapy in clinical practice.

We have demonstrated HSV-1 infection induces increased levels of soluble lipid peroxidation by-products MDA/HAE (predominantly HNE) in culture medium of neural cells at early stages of infection. The fact, that HSV-1 infection of neural cells is associated with increased release of highly bioactive lipid peroxidation by-products MDA and HNE *in vitro* raises the possibility of a novel mechanism by which HSV-1 may affect the physiology and may damage uninfected bystander cells in the nervous system *in vivo*. This observation indicates HSV-1 infection can directly cause oxidative damage in neural cells *in vitro* and suggests HSV-1 infection may contribute directly to oxidative tissue damage caused by HSE.

Thus far, we have seen HSV-1 induces oxidative stress, which can lead to oxidative damage. We also studied how oxidative damage, specifically HNE, affects HSV-1 replication. Based on results using neuronal and -neuronal cells , we propose HNE pretreatment can modify the HSV-1 replication cycle in a cell type and concentration dependent manner. These results suggest high concentrations of HNE may reduce HSV-1 replication in P19N cultures by

reducing the number of viable cells. Interestingly, results with P19 and P19N cells showed the physiological concentration of HNE effected HSV-1 replication in a cell differentiation dependent manner. HNE and MDA released from HSV-1 infected cells may not only induce cell death in uninfected bystander cells, but also modulate their permissiveness to HSV-1 infection in the nervous system. Our finding could be very important because, during occasional reactivation, the already present HNE could accelerate HSV-1 replication and help overcome the antioxidant defense system to contribute to HSE development. Our observations may lead to a better understanding of HSE pathogenesis, including the mechanisms of tissue injury and regulation of viral replication in neural tissues.

Finally, we detected a role of a small Rho GTPase, RhoA, in HSV cell-cell spread. Cell-cell spread is one of the primary ways HSV travels through the CNS. RhoA is known to be activated during HSV entry, but its role in spread is unknown. RhoA signaling, which can be activated by both nectin-1 and ROS, has shown to be associated with disruption of tight junctions leading to invasion of immune cells into the CNS. Our findings suggest RhoA activity has a direct effect on cell-cell fusion levels and formation of multinucleated syncytia. Maximum activation of RhoA occurred at an early time point, suggesting host receptors and viral glycoproteins are critical for RhoA activation and thus, play a crucial role in HSV pathogenesis. Data suggest RhoA is important in the early stages of cell-cell spread. Since activity decreased by 60min, but increased again at 2h, 3h, and 5h, suggests Rho signaling may take part in later signaling events as well or its activation occurs in a cyclical manner as new fusion events occur. Initial RhoA activation could be due to nectin-1, while later signaling events could be initiated by ROS generated during HSV infection. Because, Rho A has so many functions, RhoA activation during HSV infection could be critical for HSE development. RhoA could be crucial for more efficient HSV entry and spread, which can lead to tissue damage due to destruction of cells and increased ROS generation. ROS can use RhoA signaling to disrupt tight junctions, which are regulated by the nectin-afadin system and the blood brain barrier. This could lead to invasion of immune cells and further tissue damage. RhoA could be crucial in both direct virus-mediated (HSV cell-cell spread) and indirect immune-mediated inflammatory processes (inflammatory cell invasion supported by Rho signaling) that lead to HSE development. Altogether, this could lead to extensive tissue damage and

contribute significantly to HSE development. Though it appears ROS, and possibly nectin-1, can activate RhoA during HSV-mediated oxidative damage, further work must be done to study the signaling pathway. It is possible interaction of HSV gD with nectin-1 mimics nectin-1 trans-interactions and activates the RhoA signaling pathway.

It is also very important to mention existing neurodevelopmental theories for both HSE and schizophrenia believe early onset of the disease [146] is due to impaired lamination and failed neuronal migration. Early, perinatal virus infections increase the risk of incidence of this schizophrenia [147, 148]. According to another study, lipid peroxidation can contribute to the pathogenesis of schizophrenia [149-151]. It is known HSV infection of the CNS can be associated with a number of neurological sequelae. These sequelae include deficits in memory and executive functioning similar to those found in some individuals with schizophrenia [152-154]. Some individuals with acute HSV infection of the CNS display psychiatric symptoms such as functional psychosis [155, 156]. Evidence of HSV DNA has been found in postmortem brain samples obtained from a small number of individuals with schizophrenia [39]. Serologic studies have indicated some populations of individuals with schizophrenia have increased evidence of exposure to human herpesviruses [157, 158]. An association between reduced cognitive functioning and the prevalence of HSV-1 antibodies was found among outpatients with schizophrenia [159]. HSVs have been considered candidates that may contribute to the psychopathology and aetiology of schizophrenia. Our study has provided a critical link between HSV infection and schizophrenia. Since lipid peroxidation can contribute to schizophrenia, our results suggest lipid peroxidation could be the mechanism behind the development of HSV-induced schizophrenia.

HSV may contribute to various neurological diseases. Our findings on HSV infection may contribute to a better understanding of pathogenesis of other neurological disease, where HSV infection is an independent risk factor, and may help develop clinical therapeutic agents against them.

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VIII. ÖSSZEFOGLALÓ

A HSE a legelterjedtebb sporadikus enkefalitisz emberben, mely elégtelen vagy hatástalan antivirális kezelés mellett igen magas elhalálozási rátával bír. A kezelést követően a túlélőknek kevesebb, mint 3%-a nyeri vissza normál agyi funkcióit. Egyéves kor feletti gyermekeknél, illetve felnőttek esetében a HSV-1 felelős a HSV enkefalitisz kimagasló hányadáért, ami jellemzően egyoldali és a limbikus területeket, illetve a prefrontális régiót érinti. Míg újszülöttekben a HSE kialakulása, mely ez esetben diffúz, több agyterületet érint preferáltság nélkül, főként HSV-2 fertőzésre vezethető vissza. Napjainkban még nem teljesen tisztázott miért mutat életkortól függően jelentős különbséget mind patogenezisében, mind klinikai megnyilvánulásában az idegrendszer HSV fertőzése. Vizsgálataink rámutattak ennek a paradigmának egy lehetséges magyarázatára, mely a nectin-1 térbeni és időbeni eloszlásának változása az agy fejlődése során. A nectin-1, amellett, hogy sejtadhéziós molekula, a HSV elsődleges receptora a központi idegrendszerben. Kísérleteink során újszülött rágcsáló agyban intenzív nectin-1 expressziót figyeltünk meg a kortikális területeken, valamint a hemiszférák között lévő kapcsoló struktúrákban (kéregtest, comissura hippocampi, és commissura anterior). A nectin-1 kifejeződés a kor előre haladtával növekedett a limbikus rendszerben, és ezzel párhuzamosan csökkent az asszociációs területeken. A nectin-1 kifejeződés markáns csökkenése a kéregtestben az agy egyedfejlődése során korrelált a felnőtt agyra jellemző egyoldali HSV terjedéssel. Eredményeinkből arra következtettünk, hogy az agy fejlődése során a vírus receptor (nectin-1) térbeli és időbeli kifejeződésének változása magyarázatul szolgálhat arra, hogy mikor mennyire fogékony az agy a neurotróp HSV fertőzésre.

Az újszülött kori HSE-t olyan patológiai elváltozások kísérik, mint kérgi atrófia, vízfejűség, microphthalmia. Mely azt sugallja, hogy ezek az agyi elváltozások a vírus közvetlen pusztításának tudhatók be, másrészt, hogy különösen a vándorló neuronok vannak a támadás középpontjában. Rágcsálókon végzett kísérletek is megerősítik azt a nézetet, miszerint a fejlődő agy sokkal fogékonyabb a HSV fertőzésre. Humán és rágcsáló mintákat vizsgálva arra a következtetésre jutottunk, hogy a nectin-1 tevékeny irányítója a radiális sejtvándorlásnak, ugyanis kifejeződik mind a vándorló sejteken, mind azokon a sejteken, melyek a

sejtvándorlást irányítják (ld.: radiális gliák és Cajal Retzius sejtek). Ez alapján felállítottunk egy lehetséges modellt, arról, hogyan szabályozza a sejtek radiális vándorlását a specifikus, rétegenként eltérő nectin-1 kifejeződés. A HSV gD–nectin-1 kapcsolat kialakulása nélkülözhetetlen az eredményes vírusfertőzéshez. Így a nectin-1-hez kapcsolódó HSV gD befolyásolja a nectin-1 élettani funkcióját, mely a sejtvándorlás során a radiális glia hálózat és a vándorló idegsejtek intim kapcsolatának megszakadásához, ezáltal hibás kérgi rétegződéshez vezethet. Kísérleteink azt mutatták, hogy a radiális vándorlás mellet a nectin-1 részt vesz a tangenciális vándorlásban is. A rágcsáló szaglógumóban ennek kapcsán azonosítottunk egy új sejtvándorlási útvonalat: a CMS-t (callosalis migrációs út), mely a rostralis migrációs úttal (RMS-sel) párhuzamosan fut, és a fejlődés csak egy bizonyos stádiumában található meg. Igaz, hogy a radiális és tangenciális vándorlás között a kapcsolat még teljességében nem ismert, úgy tűnik, a nectin-1 kapcsolatot teremt a kétféle vándorlási típus között. Elmondhatjuk, hogy az agy fejlődése során a nectin-1 fontos szerepet tölt be a neuronok mind radiális, mind tangenciális vándorlásában. Így egy méhen belül bekövetkező HSV fertőzés nagymértékben megváltoztathatja mind a kérgi rétegződést, mind a kérgi összeköttetéseket. Mivel a HSV más receptorokat is használ a sejtbe való bejutáshoz, elképzelhető, hogy ezeknek, a receptoroknak az eloszlása módosíthatja a HSE patológiai megjelenését.

Az előbbiekben bemutattuk, hogy a nectin-1 eloszlásának változása milyen kritikus szerepet tölt be a HSE manifesztálódásában, de a pontos molekuláris mechanizmus, mely révén a HSV fertőzés HSE kialakulásához vezet, még felderítésre vár. Eredményeink azt sugallják, hogy a vírus által indukált oxidatív stressz és lipid peroxidáció, valamint a sejtről sejtre terjedés során aktiválódó RhoA jelátvitel, szerepet játszhat ebben a folyamatban, mind a vírus révén közvetlenül, mind az immunrendszer bevonása révén közvetetten.

Az oxidatív stressz, mint kísérőjelenséget, már sokféle vírusfertőzés esetében leírták. Kísérleteink kimutatták, hogy HSV közvetlen módon képes reaktív oxigén (ROS) és nitrogén (RNS) gyökök felhalmozódása révén oxidatív stressz kiváltására in vitro neuronális sejtkultúrán (P19N), továbbá, hogy a HSV fertőzést követő ROS képződéshez szükség van a vírus sejtbe való bejutására, valamint replikációjára. Emelt ROS szint már a fertőzést követő első órában megfigyelhető, ami arra enged következtetni, hogy az oxidatív stressz kiváltása a

bejutással egy időben, vagy közvetlen utána lezajló esemény, melyhez elegendő egy vagy több virális *„immediate early"* vagy *„early"* gén átíródása. A HSV-1 fertőzés antioxidánssal, ebselnnel, mely egy glutation peroxidáz típusú enzim, visszaszorítható. Ez a tény megint csak megerősíti azt a feltételezést, hogy az eredményes fertőzéshez a vírusnak oxidatív stresszt kell indukálnia. Az oxidatív stressz a membránok foszfolipidjeinek károsítása révén lipid peroxidációhoz vezet, melynek során olyan bioaktív melléktermékek keletkeznek, mint 4-hydroxi-2-nonenal (HNE) és malondialdehid (MDA). Kísérleteink azt mutatták, hogy HSV-1 közvetlenül indukál lipid peroxidációt, mely jelentős sejtpusztuláshoz vezet a sejtmembrán szétesése és a nukleinsav károsodása révén. Az HNE és MDA amellett, hogy apoptózist indukál, kis koncentrációban regulátor funkciót tölt be a sejtosztódás és a sejtdifferenciáció folyamatában. Munkánk során megvizsgáltuk, hogy magas illetve fiziológiás koncentrációban jelenlevő HNE hogyan hat a vírusfertőzésre. Érdekes módodon, azt kaptuk, hogy differenciált idegsejtek környezetében kis koncentrációban jelenlévő HNE pozitívan hat a vírusfertőzésre, mely maga után vonja azt a lehetőséget, hogy ezáltal a szomszédos, még nem fertőzött sejtek HSV-re való fogékonysága befolyásolható. Ez nagyon fontos lehet, ha számba vesszük, hogy az alkalmankénti reaktiváció során a látens fertőzés helyén is kimutatható enyhén emelkedett szintű HNE, mely ezáltal gyorsíthatja a fertőzés kiterjedését, és így segíti a gazdaszervezet védekező, antioxidáns rendszerének visszaszorítását. Munkánk, arra enged következtetni, hogy az oxidatív stressz és oxidatív károsodás a HSV fertőzés esszenciális velejárója, mely jelentős mértékben hozzájárulhat a HSE kialakításához.

Végül a RhoA kis GTP-áz szerepét vizsgáltuk a vírus sejtről sejtre való terjedésében CHO-K1 sejteken. Mivel a RhoA befolyásolja az idegsejtek differenciálódását, ezért úgy találtuk, hogy egy nem idegi sejtvonal alkalmazása, pontosabb következtetéseket enged levonni a mechanizmust illetően. A vírus sejtről-sejtre való terjedése, elsődleges módja annak, hogy a vírus az idegrendszeren belül újabb és újabb sejteket hajtson igába. Ismert, hogy a vírus sejtbe lépésekor RhoA aktiválódik nectin-1-t expresszáló sejtekben, de szerepe a sejtről-sejtre való terjedés során eddig a pontig feltáratlan. Kísérleteink azt mutatták, hogy az aktivált RhoA elősegíti a sejt-sejt fúziót és a szincíciumok kialakulását. A legmagasabb RhoA aktivációt a sejtek összeeresztését követő első 20 percben tapasztaltuk, mely azt mutatja, hogy a receptor és vírus

glikoproteinek jelenléte elengedhetetlen a RhoA aktivációhoz, továbbá azt, hogy a RhoA korai aktiválódása elengedhetetlen a fertőzés korai fázisában. Mivel az aktivitás az első 60 percet követően ismét megemelkedett 2h, 3h és 5h óránál, ez több dologra enged következtetni, egyrészt, hogy a RhoA részt vesz késői jelátviteli folyamatokban is, vagy aktivációja ciklikus és az újabb és újabb fúziót ismételt RhoA aktiváció követi. A kezdeti RhoA aktiváció nectin-1-hez kapcsolódó jelátvitel következménye lehet, míg a későbbi kapcsolódhat a HSV-1 által indukált ROS képződéshez. ROS képes felhasználni RhoA jelátvitelt arra, hogy feloldja a szoros kapcsolatokat (tight junction), melyek nectin-afadin rendszer szabályozása alatt állnak. A szoros kapcsolatok felbomlása a vér-agygát károsodásához vezet, mely lehetővé teszi az immunsejtek invázióját. Az így bejutó immunsejtek tevékenysége hozzájárul a ROS szintjének további emelkedéséhez. Tehát RhoA feltehetően jelentős mind a vírus által közvetlen mediált (HSV sejtről-sejtre terjedése), mind az immunrendszer által közvetített gyulladásos folyamatok kiváltásában (immunsejtek RhoA jelátvitel által történő beáramlása a fertőzés helyére). Ezek a folyamatok hozzájárulhatnak a HSE kialakulásában szerepet játszó nagymértékű sejtkárosodáshoz, és sejtpusztuláshoz. Bár úgy tűnik, hogy ROS és nectin-1 képes aktiválni a RhoA kis GTP-ázt a HSV által kiváltott oxidatív károsodás során, további vizsgálatok elvégzése indokolt, mely a jelátviteli út pontosabb megértéséhez vezethet.

Zárszóként említésre méltó, hogy HSV-1 jelenlétét herpesz enkefalitiszen kívül olyan idegrendszeri betegségekben is, mint Alzheimer kór és szkizofrénia, leírták már, bár ezekben a HSV szerepe még tisztázatlan. Mindenesetre érdekes az a többszörös egybeesés, miszerint a HSE-ben és a szkizofréniában érintett agyi területek azonosak, továbbá egy perinatális herpesz vírus fertőzés növeli a kockázatát egy esetleges, az egyedfejlődés során később megjelenő, szkizofrénia kialakulásának, valamint az is, hogy a lipid peroxidáció egyes elméletek szerint hozzájárul a szkizofrénia patológiai elváltozásaihoz. Így HSV által kiváltott idegrendszeri elváltozások mögött álló folyamatok kutatása hozzájárulhat más neurológia betegségek patológiájának tisztázásához és segíthet egy ellenük irányuló klinikai terápia kifejlesztéséhez.

IX.   KÖSZÖNETNYILVÁNÍTÁS

Elsőként szeretnék köszönetet mondani az Alma Maternek, a Szegedi Tudományegyetemnek, hogy elsősorban a Pszichiátriai, Orvos Biológiai valamint Élet és Idegtudományi Intézete által nyújtott szakszerű, alapos képzés lehetővé tette ennek a dolgozatnak a megszületését. Köszönettel tartozom témavezetőmnek, Dr. Horváth Szatmárnak, aki kezdetektől fogva támogatta tudományos előmenetelemet. Úgy érzem, a jelenségek mögötti folyamatok jobb megértéshez vezetett, az, hogy munkámat nemcsak kutatóként, hanem orvosként is felülbírálta. Lényeg látása és motivációja mindig példaként állt előttem. Külön köszönet illeti Dr Vályi-Nagy Tibort, kinek laborjában tölthettem 15 hónapot az Illinois Egyetemen, Chichago-ban, akiben nemcsak egy nagyon logikusan gondolkodó kutatóra, hanem barátra is leltem. Szeretnék köszönetet mondani Dr. Rosztoczy Ferencnek, aki lehetővé tette számomra Rosztoczy alapítványtól kapott ösztöndíj révén ezt a sok szempontból gyümölcsöző amerikai tanulmányutat. Továbbá köszönettel tartozom Dr. Deepak Shukla-nak, azért hogy lehetőséget biztosított laborjában a molekuláris munkák elvégzésére, egy újabb amerikai tanulmányút keretén belül, melyre a Magyar Oktatási Minisztérium és a Doktoranduszok Országos Szövetsége biztosította az anyagi fedezetet. És semmiképpen sem szeretném a sorból kihagyni Dr. Toldi Józsefet, akire mindig minden körülmények között számíthattam, annak ellenére, hogy már nem tartoztam hivatalosan az Intézetéhez.

Szeretném megköszönni az összes kollégának, a teljesség igénye nélkül Dr. Seprényi Györgynek, az Orvos Biológiai Intézet asszisztenseinek, az Élettani Intézet munkatársainak meg nem szűnő barátságát és támogatását.

Nem utolsó sorban, de a legnagyobb köszönettel a szüleimnek, öcsémnek és jövendőbeli páromnak tartozom. Köszönöm a türelmet, a biztatást, azt hogy mindig büszkék voltatok rám. És nem utolsó sorban nektek, Marcsi, Rita, Dóri, Andi, kedves barátok, akikre mindig számíthattam Nélkületek, ezt nem tudtam volna véghez vinni.

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