Journal of NeuroVirology, 14: 164–172, 2008

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280801898672

informa
healthcare

Short Communication

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

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Herpes simplex viruses (HSV) produce age-dependent encephalitis characterized by more severe involvement of the cerebral cortex in younger hosts. To elucidate the potential role of the major neural entry receptor of HSV, nectin-1, in age-dependent susceptibility of cortical neurons to viral encephalitis, the authors examined the anatomical distribution of the receptor protein in the developing human and mouse cerebral cortex, hippocampus, and cerebellum by immunohistochemistry. Nectin-1 is expressed at high levels in guiding cells (radial glial cells and Cajal-Retzius cells) that regulate radial migration of neurons in cortical lamination, at lower levels in migrating neurons, and at variable levels in the transient ventricular and marginal zones of the cerebral cortical wall. These results may have implications regarding the selective spatiotemporal tropism of HSV to specific neuronal populations, and for the better understanding of neurodevelopmental defects caused by fetal HSV infections. *Journal of NeuroVirology* (2008) **14,** 164–172.

Keywords: Cajal-Retzius cell; herpes encephalitis; neurotropism; radial glia; susceptibility

Introduction

Viral encephalitis due to infection with herpes simplex viruses (HSV) including HSV-1 and HSV-2 is an important cause of mortality and often results in significant long-term neurological deficits in survivors (Kennedy and Chaudhuri, 2002; Tyler, 2004a, 2004b). During fetal and neonatal HSV-1 and HSV-2 infections, encephalitis can follow transneural or viremic spread of virus to the nervous system and congenital infections may cause central nervous system (CNS) malformations.

Neurons are the primary target cells for HSV, and the outcome of infection is determined by both host and viral factors. An important host determinant is the maturity of the neuronal population infected (Lennette and Koprowski, 1944). In mice, HSV causes the death of immature neurons, whereas mature neurons can often survive the infection (Kristensson, 1976; Yamamoto *et al*, 1967). An affinity of HSV to immature neurons has also been observed following HSV infection of organotypic brain slice cultures (Braun *et al*, 2006). Increasing age correlates with reduced virus replication in the brain and reduced virus-induced mortality; this reduced susceptibility

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This work was supported by the research grants OTKA (T046687), GVOP-3.2.1.-2004-04-0357/3.0 and RET-08/04. S.H. held a Bolyai and Öveges scholarship and E.P. and S.K.K. were Rosztóczy Foundation scholars during part of this project. The financial support from these foundations is gratefully acknowledged.

Received 13 June 2007; revised 19 November 2007; accepted 29 December 2007.

of the mature brain may only be in part due to maturation of the immune system and the blood-brain barrier (Yamamoto *et al*, 1967). The susceptibility of immature neurons to HSV may be related to an increased expression of neuronal surface molecules that act as receptors for viral entry. Indeed, age-dependent changes in the pattern of expression of viral entry receptors may play a critically important role in the determination of age-dependent susceptibility to HSV.

In mammals, including humans, neurons of the cerebral neocortex, similarly as in the other laminated structures of the CNS such as the cerebellum, are generated during fetal life in the proliferative zones and then migrate radially to their final destinations (Rakic, 1988). The migration of these immature neurons depends on guidance mechanisms and may need a set of molecules to reach their proper areal and laminar destination (Rakic, 2006). These guiding molecules often serve as cell surface receptors for attachment and entry of several pathogens (Fazakerley, 2001).

Entry of HSV into neurons is a multistep process depending upon the interaction of a variety of cell surface receptors and viral envelope glycoproteins (Schweighardt and Atwood, 2001). Nectin-1 (N1) is a cell surface adhesion molecule that mediates the entry of both HSV-1 and HSV-2 through interaction with the HSV envelope glycoprotein D. N1 has been shown to be the primary receptor for HSV-1 entry into human fetal neurons (Mata *et al*, 2001; Simpson *et al*, 2005).

Gene therapy using replication-competent HSV for the treatment of primary brain tumors has proven to be a promising novel treatment modality. Recent attention has been focused on the potential of replication-competent viruses to discriminatingly target, replicate within, and destroy tumor cells via oncolysis, leaving adjacent neurons unharmed. However, a much recent work of Radbill and coworkers revealed frequent unilateral ventriculomegaly in newborn animals after the administration of a replication-competent HSV with tumor cell–specific lytic activity (Radbill *et al*, 2007). One plausible reason is that immature migrating neural cells that express similar cell surface markers are affected by tumor cell–specific lytic activity (Reya *et al*, 2001).

We have recently shown the expression of N1 in normal adult and neoplastic human nervous system tissues (Guzman *et al*, 2006). Moreover, N1 expression was found to be a good predictor of oncolytic HSV sensitivity in primary brain tumor samples (Rueger *et al*, 2005). We also described in a murine model, that N1 expression is developmentally regulated and altered by HSV-1 infection in the CNS, which may contribute to HSV-induced pathology and dissemination (Horvath *et al*, 2006; Shukla *et al*, 2006). Therefore we suggest that the understanding of the changes in the distribution of N1 during normal human brain development can help avoid adverse events of HSV-based oncolytic therapy in children.

The aim of the present study was to determine the anatomical distribution of the N1 in the developing human cerebral cortex, hippocampus, and cerebellum and to correlate N1 expression in the human immature brain to model murine brain according to the affinity of HSV to specific neuronal populations. The peak of the migratory phase of cortical neurons in the human fetus lasts 18 times longer than in mice (between 6 and 24 weeks of gestation), compared with the duration of only 1 week (between embryonic days E11 and E18) in the mouse. Due to the limited tissue availability, in our experiments we have studied N1 expression during neuronal migration in human fetal brain tissues at approximately 20 weeks of gestational age and in newborn (E18) murine brain tissues.

Results

Nectin-1 expression in human corneal and uterine tissues

First, human corneal and uterine tissues were analyzed to determine whether immunohistochemical techniques used in this study were capable of N1 detection in a pattern consistent with that previously reported (Guzman *et al*, 2006; Mata *et al*, 2001; Shukla *et al*, 2006; Simpson *et al*, 2005). N1 immunostaining was observed in the glands of human endometrial tissue, whereas endometrial stroma did not express this protein (Figure 1B). Human cornea showed widespread N1 expression among cells of the corneal epithelium and no significant nectin-1 expression in the corneal stroma (Figure 1D). As a negative control, immunohistochemical analysis performed without primary antibody did not yield staining of human cornea or human endometrial tissues (Figure 1A, C). These findings are consistent with previously reported observations concerning N1 expression in various cell types, and indicate that the immunohistochemical assay used here has sufficient sensitivity and specificity to detect nectin-1.

Nectin-1 expression in fetal human and murine neocortex

Next, we analyzed N1 expression in developing nervous system tissues. N1 was strongly expressed in the leptomeninges. In the most superficial, marginal zone of the human fetal neocortex, we observed N1-positive neurons with the morphologic appearance of Cajal-Retzius (CR) cells (Figure 2A, D). N1 staining was detected in these cells around the nucleus and in cell processes. Most cells were bipolar, though some had more than three processes (Figure 2G). The intensity of the staining was variable, possibly reflecting differences in fixation and postmortem interval of the autopsy specimens.

In the deeper cortex, N1 immunoreactivity of neurons increased as a function of the distance from the pial surface: N1 positivity was detected in the cytoplasm and processes of neurons in the deeper layers

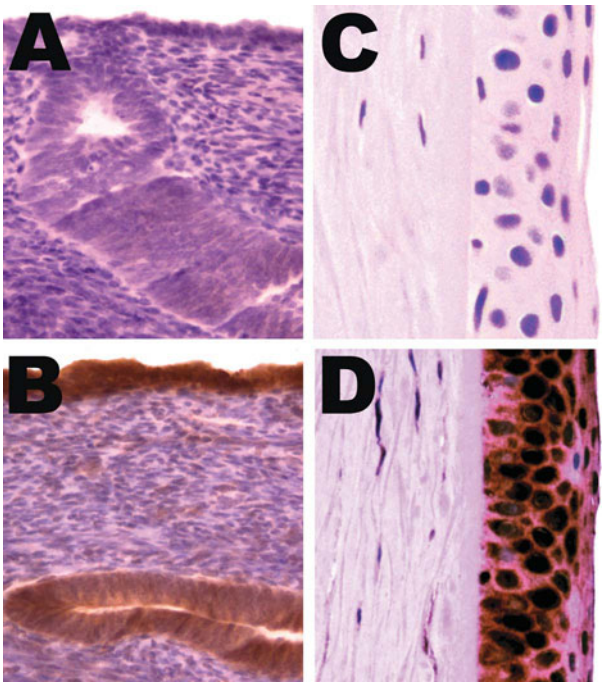

Figure 1 Immunohistochemical analysis performed without primary antibody did not yield staining of human cornea or human endometrial tissues (**A, C**). (**B**) N1 immunostaining was observed in the glands of human endometrial tissue, whereas endometrial stroma did not express this protein. (**D**) Human cornea showed widespread N1 expression among cells of the corneal epithelium and no significant nectin-1 expression in the corneal stroma.

of the cortex but was essentially absent in more superficial neurons (Figure 2B, C). N1 staining was also detected in subcortical migratory neurons (Figure 2E, H, I, and J). In the germinal layer, variable N1 immunoreactivity was detected, ranging from very weak to no N1 expression in cells close to the ventricular surface to N1 positivity in deeper positioned cells (Figure 2C, F). The ependymal layer was strongly N1 positive (Figure 2K).

Similarly to the human brain, in the newborn mouse cortex the highest N1 signal was associated with the cerebral cortex (Figure 3A). The transient strata: ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), and subplate (SP), had highly specialized N1 staining pattern in the newborn murine brain.

N1 immunoreactivity was prominent in the upper tiers of the cortical plate just below the marginal zone with N1-positive neurons with the morphologic appearance of Cajal-Retzius cells (Figure 3B). Radially orientated apical dendrites of N1-positive neurons indicated a predominant pyramidal phenotype.

N1 was expressed also in the migrating cells in the intermediate zone and in neurons in the cortical plate, as well as in 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 en route to the cortical plate. N1 protein was observed in cell somata and processes, and in the horizontal fiber tracts characteristic of tangential dispersion of migratory cells within the intermediate zone (Figure 3A).

N1 protein immunosignal was hardly detected in a subset of cells in the VZ. These cells had large oval somata, and they lay close to the ventricular surface. N1 immunoreactivity was preferentially concentrated at the ventricular pole of the cytoplasm and in the endfeet lining the ventricle (Figure 3C). Despite the cloud-like N1 immunostaining pattern around these cells, we could assume that the position and shape of these N1-positive cells are characteristic to radial glia like cells. The concentration of N1 protein in ventricular endfeet and initial portions of radial processes of ventricular-zone cells points to a possible involvement of N1 in guidance of neuronal migration.

These findings indicate that N1 is widely expressed in the fetal murine and human cortex and suggest that the distribution and intensity of the N1 immunoreactivity reflects the order of appearance and the degree of maturation of neurons. Furthermore, these findings suggest that N1 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 N1 was demonstrated as small, granule cell–like migrating neurons at stratum radiatum. In addition, moderate to strong immunoreactivity for N1 was also found as traversing postmitotic principal pyramidal cells at stratum oriens (Figure 4A). In the postnatal day 7 (P7) and adult hippocampal formation N1 protein was strongly detected in the hippocampus and in the entire dentate gyrus (Figure 4B, C). The signal for N1 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 N1 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 N1 was detected at strata radiatum and oriens.

In the human fetal hippocampal formation, the subgranular layer of the dentate gyrus stained very weakly suggesting that the newly generated neural precursors do not express the entry receptor for HSV, whereas cells in the granular layer were strongly positive. The pyramidal cells of the hippocampus were strongly N1 positive (Figure 4D to F).

This pattern of protein distribution suggests that 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.

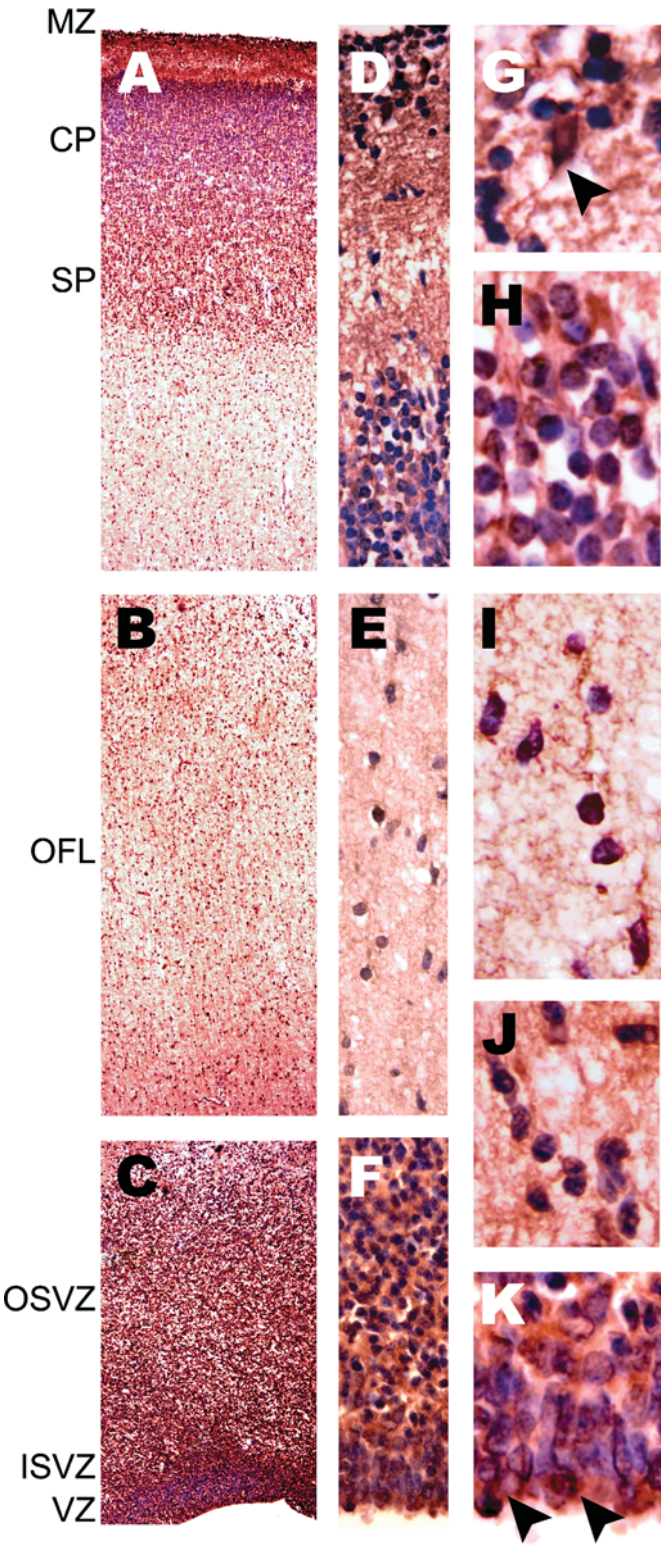

Figure 2 Immunohistochemistry for nectin-1 in fetal human cerebral cortex at 21 GW. Bright field images represent N1-specific staining of the fetal human cerebral cortical wall (**A, B, C**). Cajal–Retzius cells (*arrow*) and subpial granular neurons are strongly nectin-1 positive (**D, G**), whereas subplate neurons are N1 negative surrounded by N1-positive fibers (**H**). As a part of the intermediate zone (**E**), an early appearing outer fibre layer (OFL) forms a N1-positive landmark during development (**I, J**). N1 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 N1-specific staining in **H, I, J**). N1 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 N1 (**J**). CP, cortical plate; MZ, marginal zone; SP, subplate.

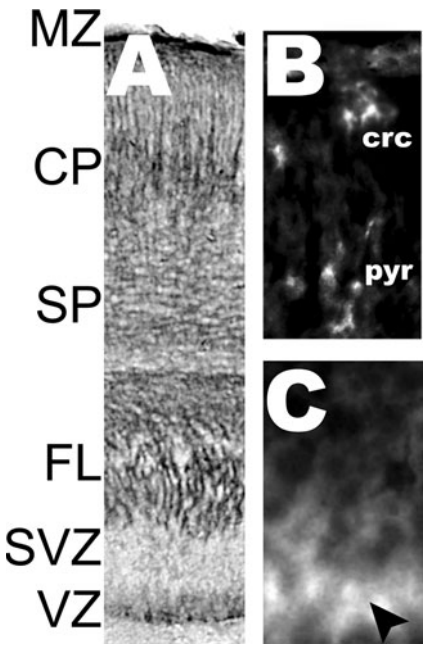

Figure 3 Nectin-1–specific immunolabeling in the newborn mouse cortex (**A**). N1 was expressed in the migrating cells in the intermediate zone and in neurons in the cortical plate, as well as in cells of the ventricular zone. (**B**) N1-specific immunofluorescent signal was prominent in the upper tiers of the cortical plate just below the marginal zone with N1-positive Cajal-Retzius cells (crc) and radially orientated N1-positive pyramidal neurons (pyr). (**C**) Cloud-like N1 immunostaining pattern around radial glia-like cells in the ventricular zone (*arrow*) with N1-negative stellate-shape in the upper part.

Nectin-1 expression in fetal human and murine cerebellar cortex

Both in the fetal human and in the P0 murine cerebellum, prominent N1 immunopositivity was detected in migrating Purkinje and granular cell precursors in the deep cerebellar white matter and in these cells at their final position, in the stratum ganglionare. The entire thickness of the germinal layer was stained all along the folia, and some labeled cells were also seen in the white matter (Figure 5A, D). These data suggest a critical role of N1 protein in the tangentoradial migratory process of deep Purkinje neuroblasts to the stratum ganglionare. Similar, but only radial N1 oriented movement of external germinal layer (EGL) neuroblasts to the internal germinal layer (IGL) is also suggested.

During the first postnatal week, an intensive proliferative activity of the N1-positive neuroblasts increases the width of the external germinal layer. From the stage P7 (Figure 5B), another pattern emerged, the postmitotic granule neurons traversing the molecular layer toward the internal germinal layer, with expression in elements located, mostly in the external germinal layer (later molecular layer), suggesting that N1 was present along the dendrites of Purkinje neurons. In the adult cerebellum, the most intense immunosignal for N1 was detected in the Purkinje cell layer (Figure 5C, E). A similar developmental change of the subcellular distribution of N1 was observed in the hippocampal pyramidal cell layer.

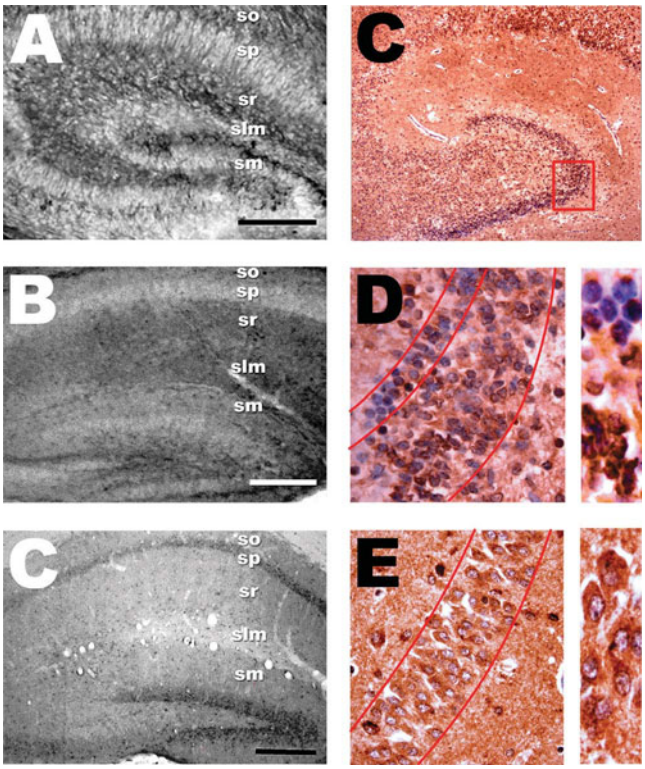

Figure 4 Developmental dynamics of nectin-1–positive fibre tracts and migrating neurons in the hippocampal formation. (**A**) On P0, nectin-1–positive bipolar cells are densely packed near the hippocampal fissure in the molecular layer of the dentate gyrus and the hippocampal stratum lacunosum moleculare. Multipolar immunoreactive cells are observed mainly in the strata oriens and radiatum, where cell bodies and thin dendrites are labeled. A few pyramidal cells are also stained; within the sp, the cell bodies and the apical dendrites are lightly immunoreactive. (**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 N1. The area with red borders indicate the region for higher magnification in **E** and **F**. (**E**) In the human fetal hippocampal formation, the subgranular layer of the dentate gyrus stained very weakly, whereas cells in the granular layer and the pyramidal cells were strongly positive. (**F**) The N1-negative subgranular layer is no more detectable in the adult human dentate gyrus. slm = stratum lacunosum moleculare; sm = stratum moleculare; so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum. Scale bars denote 200 μm.

Discussion

Nectin-1, brain morphogenesis, and HSV susceptibility

It has been known for a long time that in rodents there is a marked change in susceptibility to neurotropic

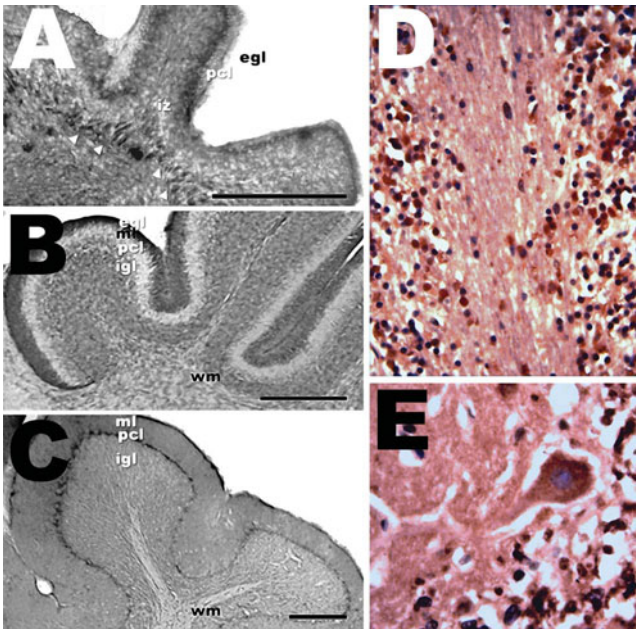

Figure 5 Three stages of development in the cerebellar cortex as revealed by nectin-1 immunohistochemistry. (**A**) In the early postnatal developmental period (on P0), nectin-1–positive Purkinje cell precursors (white arrowheads) become postmitotic and migrate from the deep cerebellar layers through the wall of the cerebellar anlage in a morphogenetic movement to their final destination. (**B**) From P7, nectin-1–positive postmitotic granule neurons traverse the ml toward the igl. (**C**) In the adult, the pattern of nectin-1 connections of the Purkinje cells and the granule neurons is established in the molecular layer and in the Purkinje cell layer. (**D**) Prominent N1 immunopositivity is detected in migrating Purkinje and granular cell precursors in the fetal human cerebellar white matter. (**E**) Purkinje cells show moderate staining for N1 in perikarya and proximal dendrites in a human sample. igl = internal germinal layer; iz = intermedier zone; ml = molecular layer; pcl = Purkinje cell layer; wm = white matter. Scale bars denote 250 μm.

virus infection during the first few postnatal days (Fazakerley, 2001). Under evolutionally pressure of natural selection, neurotropic viruses target the transcriptionally/translationally active subset of neuronal precursors. Here we demonstrate the existence and change in distribution of special population of immature neural migratory cells with the marker of the main HSV entry receptor N1. 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. HSVs (actually all CNS pathogens) use the intercellular structural and signalization cascade to enter the host neuron and replicate. The basis for that processes is molecular mimicry.

We have shown here that N1—the major entry receptor of HSV—is widely expressed in the immature human and murine brain. N1 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 the ventricular zone are N1 positive. Interestingly, the undifferentiated cells in the ventricular zone and neurons in the outermost areas of the fetal neocortex were N1 negative. Importantly, we detected strong N1 expression in a subset of cells that regulate migration of neurons, including CR neurons and radial glia cells.

Neonatal herpes simplex encephalitis 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 (Kristensson, 1976). An increased susceptibility of the immature brain to HSV was also observed in the *ex vivo* organotypic brain slice infection system (Braun *et al*, 2006). 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 in neonatal mouse and neonate rat brain. Neonate brain tissues were much more permissive for HSV-1 infection than adult mouse brain tissues (Braun *et al*, 2006). 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 data raise the possibility that the susceptibility of immature neurons to HSV may be related to an increased expression of neuronal surface molecules acting as viral receptors.

Our results on expression of the HSV receptor N1 at the protein level partially support this hypothesis. We have identified N1-positive neuronal and other cell populations in the neocortex, hippocampus, ventricular zone, and ependymal layer of the immature brain. Interestingly, the undifferentiated cells in the ventricular zone and neurons in the outermost areas of the fetal neocortex were N1 negative. Importantly, we detected strong N1 expression in a subset of cells that regulate migration of neurons, including CR neurons and radial glia cells.

N1 is expressed at high levels in neural subtypes that regulate radial migration

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 and cerebellar 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 (Rakic, 2006).

The 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.

A very recent work revealed frequent unilateral ventriculomegaly in newborn animals after the administration of a replication-competent HSV with tumor cell–specific lytic activity. Moreover, there was a close corelation between the oncolytic HSV sensitivity and N1 expression in primary brain tumor samples (Rueger *et al*, 2005). This suggests a cross-reactive lytic activity between brain tumor cells and normal neuroblasts, which share nectin-1 as a major cell surface adhesion molecule.

Our results thus demonstrate the changes in N1 expression occurring 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 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. Finally, similar radial migration occurs in the cerebellar cortex. The distribution of the N1 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 N1 expression in neurons of the immature brain, which has led us to develop the following model of radial migration: N1 is not expressed in the most immature neuroblast but becomes expressed in immature neurons that are about to begin migration. N1-expressing guiding cells send radial processes and these guiding processes are needed to lead the migratory cells. Once migrating neurons reach their intended position, N1 is again expressed to augment synaptogenesis (Figure 6).

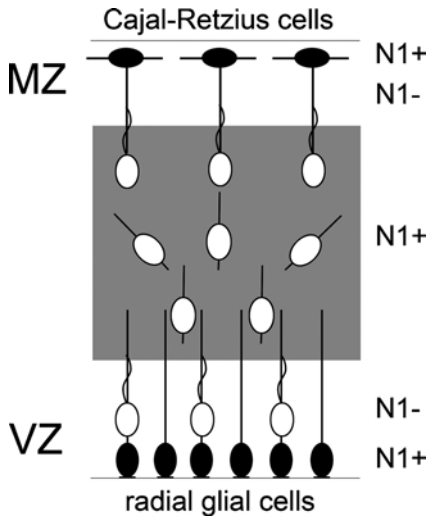

Figure 6 A model on nectin-1–sensitive neuronal migration in the mammalian neocortex. In the ventricular zone, immature neuroblast are attached to N1-positive radial glia and advance towards the CP via glia-guided locomotion. In the next step of their migration, they detach from the glia fibers and migrate tangentially under the influence of N1. N1 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, N1 is expressed again to augment synaptogenesis.

An integrative approach to understand herpes simplex infection–induced neural malformations

Herpes simplex virus types 1 and 2 are members of the neurotropic subfamily (alphaherpesviruses) of the herpesvirus family that cause acute HSV encephalitis (HSE). HSV-1 accounts for more than 90% of childhood and adult cases of HSE. In contrast, HSV-2 is responsible for most neonatal and occasional adult cases of HSE. 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 HSV 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 (Kimberlin, 2004). The pathological manifestations of intrauterine HSE are cortical atrophy, hydrocephalus, microcephaly, periventricular leukomalacia, and microphthalmia (Hutto *et al*, 1987). These malformations of the brain are suggested to be the result of the direct viral destruction of the CNS. However, the recent results in animal models of nectin malfunction show similar disturbances in eye and periventricular brain development (Honda *et al*, 2006; Inagaki *et al*, 2005). These findings and our results presented here can lead to an explanation in which 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, the HSV envelope gD influences nectin-1 function, leading to an 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 results underscore 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 the final arrangement of its vertical organization and cellular connectivity.

Whereas HSV-2 is the predominant cause of HSE in newborns, HSV-1 accounts for the vast majority of HSE in children (above 1 year old) and adults. Both types of viruses use nectin-1 as a receptor and

gD-1 and gD-2 have similar affinity for nectin-1 (Geraghty *et al*, 1998; Krummenacher *et al*, 1999). Both HSV-1 and HSV-2 can use an additional receptor, HveM (Schweighardt and Atwood, 2001). Moreover, some but not all HSV-1 isolates from CNS were able to infect cells expressing nectin-2, a receptor not used by wild-type HSV strains (Krummenacher *et al*, 2004; Warner *et al*, 1998). In addition to these proteineceous entry mediators, 3-*O*-sulfated heparin sulfate serves as a gD receptor specific for HSV-1 (Shukla *et al*, 1999). Thus, in addition to the distribution of nectin-1, it is possible that the type and distribution of other potential receptors may modify the pathological picture of the disease, depending on the type of HSV. The widespread expression of N1 in brain regions known to have increased susceptibility to HSV infection in the immature brain suggests that N1 expression plays a critically important role in regulating HSV pathogenesis in the fetal and neonatal brain. Particularly important could be our finding that N1 is expressed in cells important for the guidance of migrating neurons. N1 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. Further research in this field is required.

Materials and methods

Animals and surgical procedures
The experimental procedures used in this study 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 in the morning. A positive vaginal plug was considered to indicate the day of conception, embryonic day 0 (E0). Birth usually occurred on day E19 or E20. In order 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 the time of birth of litters. Pups and adults were deeply anaesthetized (ketamine 10.0 mg/100 g and xylazine 0.8 mg/100 g body weight intraperitoneal [i.p.]) and perfused transcardially for 5 to 10 min with ice-cold phosphate-buffered saline (PBS; 0.1 M, pH 7.3), followed by Zamboni's fixative for the same time (2.0% aqueous paraformaldehyde solution (from a 16% stock solution containing 15% picric acid) in 0.1 M sodium phosphate buffer stock, pH 7.3). The brains were dissected out and cut with a razor into coronal sections (2-mm thickness), which were soaked with the same fixative at 4°C for 2 h. For cryoprotection, sections were placed into 20% sucrose solution for 2 h and then 25% sucrose solution overnight, after which they were embedded in OCT (Optimum Cutting Temperature media; Miles, IN), and frozen with liquid nitrogen. They were then cut on a cryostat at 15 to 20 μm in the coronal plane.

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. The use of these tissues was approved by the Institutional Review Board of the University of Illinois at Chicago.

Immunohistochemistry
The primary antibodies used in this study and their dilutions were rat monoclonal anti-mouse nectin-1 (clone 48-12), which recognize the extracellular domain (courtesy of Professor Yoshimi Takai, Osaka University Graduate School of Medicine, Suita, Japan), 1:2; rabbit anti-human nectin-1 (R166), 1:1000. The rat anti-nectin-1 monoclonal antibody (mAb) (clone 48-12) was prepared and the specificity was confirmed as described by Takahashi (Takahashi *et al*, 1999) and shows reactivity against mouse and rat neurons (Horvath *et al*, 2006; Inagaki *et al*, 2003). The rabbit polyclonal serum R166 (courtesy of Professors Roselyn J. Eisenberg, Gary H. Cohen and Claude Krummenacher, University of Pennsylvania, Philadelphia, Pennsylvania, USA) raised against human nectin-1 ectodomain shows reactivity against nectin-1 in human and rat neurons (Horvath *et al*, 2006; Shukla *et al*, 2006; Simpson *et al*, 2005). The secondary antibodies used and their dilutions were biotinylated anti-rat immunoglobulin G (IgG) (H + L), mouse adsorbed, made in rabbit (Vector Labs), AMCA-conjugated anti-rat IgG (H − L) made in donkey (Jackson Lab), 1:200; biotinylated anti-rabbit IgG (H + L), made in goat (Vector Labs), 1:200.

Immunolabeling was revealed by the avidin-biotin-HRP (horseradish peroxidase) method (VECTASTAIN Elite ABC; Vector Labs). Sections were washed in 0.01 M PBS, pH 7.4, reincubated for 60 min with blocking serum (10% normal goat serum, 0.01% Triton X-100 in 0.01 M PBS, pH 7.4,) and then incubated with a primary antibody diluted in the same blocking serum. Incubation times were dependent on temperature. At 4°C, sections were incubated overnight with the primary antibody. Sections were next washed with PBS, and incubated for 1 to 2 h with the secondary antibody diluted in blocking serum. Sections were rinsed with PBS and then exposed to the avidin-biotin-HRP complex for 30 to 40 min, and the HRP was visualized with diaminobenzidine. Sections were mounted on Superfrost microscope slides (Fisher), dehydrated, and coverslipped with Entellan (Merck). For control sections, the primary antibody was omitted or preimmun sera were used. No labeling was found in the controls. The slides were processed digitally (Olympus BX51, DP70, and Olympus DP Manager).

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