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Spatiotemporal changes of the herpes simplex virus entry receptor nectin-1 in murine brain during postnatal development

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Herpes simplex virus (HSV) is known to replicate within the limbic system and to alter behavior in both humans and experimental animals. However, the reason why the virus selectively damages this anatomical, developmental, and functional neural unit remains a mystery. Nor is it known why herpes simplex encephalitis fails to respect these neuroanatomical boundaries in newborns. In the present study, the authors determined the spatiotemporal changes in the distribution of the major neural entry receptor for HSV (nectin-1) in postnatal mouse and rat brains. Discrete nectin-1 immunopositivity was observed in regions susceptible to HSV infection in specific developmental phases of central nervous system. The authors also describe nectin-1–related pathways controlling neuronal cell migration/brain morphogenesis, the disruption of which might lead to the emergence of mental disorders with a rapid cognitive decline. *Journal of NeuroVirology* (2006) **12,** 161–170.

Keywords: Alzheimer's disease; corpus callosum; herpes encephalitis; neural migration; schizophrenia

Introduction

Herpes simplex virus (HSV) 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 HSE results from the disseminated HSV-2 infection in the newborn acquired during the genital passage at the time of delivery (Chaudhuri and Kennedy, 2002). In newborns with disseminated infection, HSV-2 can reach the central nervous system (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 (Kimberlin, 2004; Whitley, 1990). 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 the survivors returning to a normal function (Whitley, 1990). With the advent of safe and effective antiviral (acyclovir) therapy for treating HSE, significant improvements in

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mortality and morbidity have been achieved (Whitley and Kimberlin, 1999). The mainstay of effective therapy against herpesviruses is the nucleoside analogue acyclovir (Whitley *et al*, 1977). Unfortunately, acyclovir therapy decreases only marginally the neurological impairment among survivors of HSE (Whitley and Kimberlin, 1999). These neurological sequelae include major defects of memory and affect, 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 (Beers *et al*, 1995).

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 (Esiri, 1982; Hierons *et al*, 1978; Kapur *et al*, 1994). In an experimental mouse model of HSE, neurohistopathological and MRI abnormalities involve predominantly limbic structures, a pattern that mimics the human disease (Meyding-Lamade *et al*, 1999). It is not known why the neuropathological pattern of HSE is characteristic of this specific set of neural structures. Nor is it known why HSV infections of the CNS display marked differences in pathogenesis and clinical presentation between newborns and adults (Buff *et al*, 1994; Corey *et al*, 1988; Jordan and Enzman, 1991; Whitley, 1991). The MR imaging of children and adults with HSE usually reveals infection of the medial temporal lobes, inferior frontal lobes, and insula. Viral spread is mostly unilateral, but spread to the contralateral side is occasionally seen at later stages of the illness, especially in the absence of therapy (Buff *et al*, 1994; Jordan and Enzman, 1991). The MR imaging characteristics of neonatal HSE vary more than do those in older children and adults, with the involvement of the white matter of both cerebral hemispheres (Corey *et al*, 1988; Jordan and Enzman, 1991; Leonard *et al*, 2000).

The contributions of viral and host factors influencing the spread of HSV in the CNS have been the subject of intense research during recent decades. HSV infects cells through an initial attachment of the virion to the plasma membrane and subsequent fusion of the viral envelope with the plasma membrane, or through direct spread between contiguous cells. These processes require several viral envelope glycoproteins (Spear and Longnecker, 2003). The viral envelope glycoprotein D (gD) can mediate entry of HSV-1 by interacting with a specific cellular receptor such as nectin-1 (Cocchi *et al*, 1998; Krummenacher *et al*, 1998, 1999; Rux *et al*, 1998). Nectin-1, also called herpes virus entry mediator C (HveC), is a receptor for HSV-1, HSV-2, as well as pseudorabies virus and bovine herpes virus (Geraghty *et al*, 1998; Warner *et al*, 1998). Although HSV can use alternate receptors such as Herpes Virus Entry Mediator (HVEM) and modified heparan sulfate (Montgomery *et al*, 1996; Shukla *et al*, 1999), nectin-1 appears to be the primary receptor for HSV-1 and HSV-2 infection of neurons in rodents and humans (Geraghty *et al*, 1998; Mata *et al*, 2001; Richart *et al*, 2003; Simpson *et al*, 2005).

Nectin-1 is a Ca^{2+}-independent cell-cell adhesion molecule from the immunoglobulin superfamily (Sakisaka *et al*, 2001). Defects in nectin-1 are linked to several forms of ectodermal dysplasia including Zlotogora-Ogur syndrome, which is characterized by cleft lip/palate, syndactyly and mental retardation (Suzuki *et al*, 2000). The expression of nectin-1 transcripts in cells of the adult mouse nervous system has been assessed by *in situ* hybridization. Specific signals have been observed in neurons of the cerebral cortex, hippocampus, dentate gyrus, and olfactory bulb (Haarr *et al*, 2001). Nectin-1 is also involved in the formation of synapses between the mossy fiber terminals and the dendrites of pyramidal cells in area CA3 of the adult mouse hippocampus (Mizoguchi *et al*, 2002; Takai *et al*, 2003).

Together, these data suggest that nectin-1 might be a key determinant for HSV spread in the CNS. The precise distribution of nectin-1 expression during postnatal murine brain development has not been reported.

In the present study we determined the spatiotemporal changes in the distribution of the major neural entry receptor for HSV (nectin-1) in postnatal mouse and rat brains. By analyzing specific phases of central nervous system development and specific regions of the brain, we observed discrete areas of nectin-1 expression in regions susceptible to HSV infection. We also describe nectin-1–related pathways controlling neuronal cell migration/brain morphogenesis.

Results

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. We used an anti-nectin-1 monoclonal antibody to detect the distribution of the receptor throughout the brains of mice as described in Materials and Methods.

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 (Figure 1A, 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

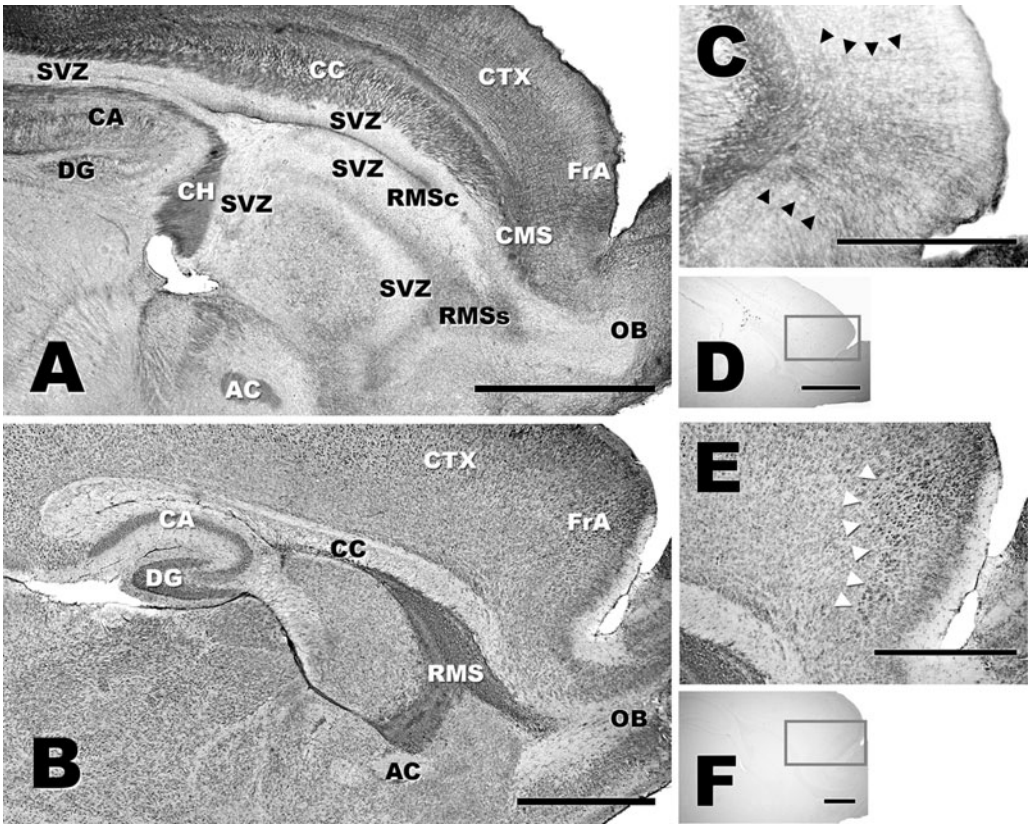

Figure 1 Nectin-1 immunostaining of paramedian sagittal sections of newborn (**A**) and adult (**B**) murine brains, respectively **A**: A prominent nectin-1 signal can be detected in the interconnecting structures of the hemispheres (CC, CH, AC). Arrays of nectin-1–positive radially oriented migrating 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. Nectin-1 was detected strongly 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 with red borders indicate the control regions for higher magnification in **C** and E. 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 scale bars on the photomicrographs **A**, **C**, and **E** denote 1 mm and 500 μm on **D, F**.

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, the olfactory bulb, and the RMS (rostral migratory stream), remained high, whereas expression in nonlimbic regions decreased to low levels (Figure 1B, 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) (Figure 1B). 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.

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

The RMS, described by Rousselot *et al* (1995), 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 the literature extended this notion, without appropriate criticism, to a similar phenomenon in every developmental stage. Here we determined 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 (Figure 1B). 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 clearly indicates that the RMS is located under the corpus callosum and is not a continuation of it.

In contrast, the nectin-1 immunopositivity of the same area of the neonatal mouse brain was only weak (Figure 1A). Chains of small cells displaying fusiform morphology could not be detected. Nevertheless, nectin-1 was strongly stained in the migrating cells of the corpus callosum and the intermediate zone of the developing cerebral cortex (Figure 2A). These arrays of migrating cells move along a RMS-like pathway, but in a slightly dorsal layer (in the zone of the corpus callosum to the external layers of the developing olfactory bulb) and a step earlier in the development (postnatal days 0 to 4; P0 to P4) than the real RMS (P7 to adult). Accordingly, we name this pathway the CMS (callosal migratory stream).

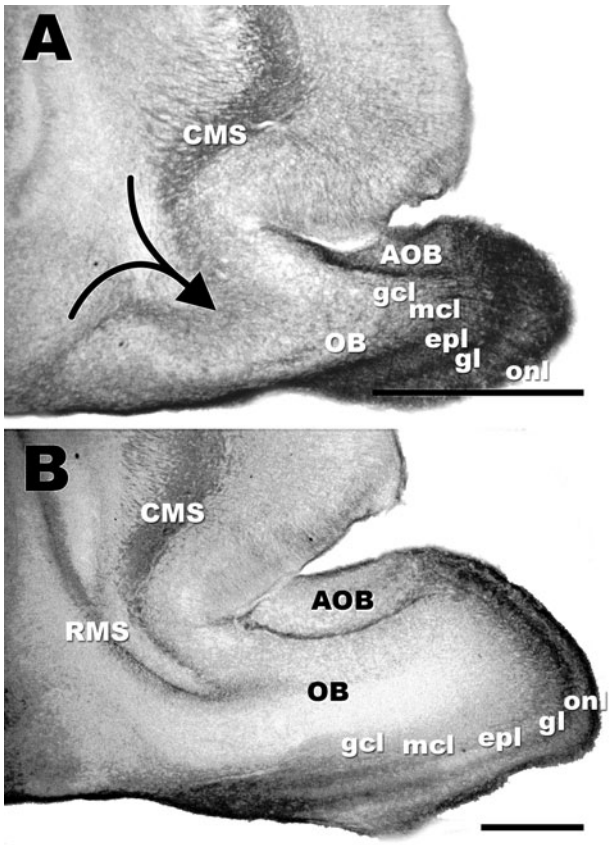

Figure 2 Comparative analysis of nectin-1 distribution in the developing olfactory system of the newborn mouse and rat. **A:** The nectin-1 immunopositivity of the RMS was only weak on P0 in the mouse. Chains of small cells displaying fusiform morphology could not be detected. Nectin-1 was detected in 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. AOB, accessory olfactory bulb; epl, external plexiform layer; gcl, granule cell layer; gl, glomerular layer; mcl, mitral cell layer; OB, olfactory bulb; onl, olfactory nerve layer. Scale bars denote 1 mm.

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. For this purpose we used a polyclonal anti-human nectin-1 antibody known to react with rat nectin-1 due to high conservation of nectin-1 between species (Ida-Hosonuma *et al*, 2003; Mata *et al*, 2001; Shukla *et al*, 2000). 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 both in the RMS and in the CMS (Figure 2B).

Nectin-1 localizes to the intermediate zone and the cortical plate but is poorly detected in the proliferating zone of the cerebral cortical wall

In the newborn mouse cortex, nectin-1 was expressed in the migrating cells in the transient intermediate zone, in neurons in the cortical plate, and in the cells in the ventricular zone (Figure 3A).

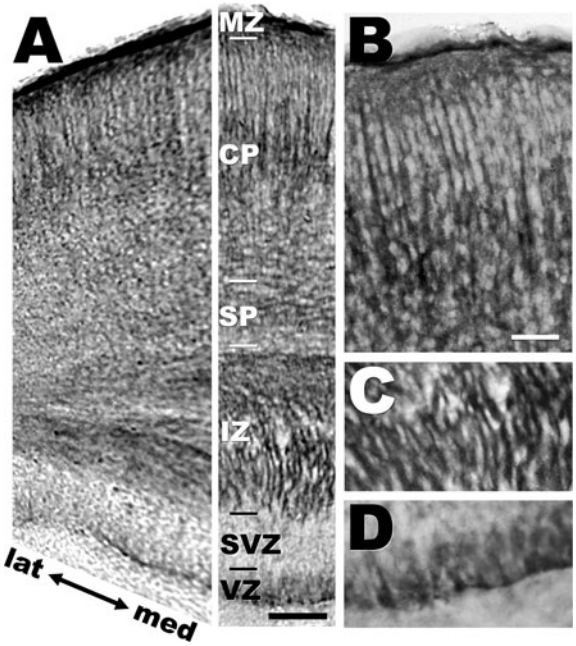

Figure 3 Nectin-1 distribution in the developing cerebral cortical wall. **A:** Three-dimensional reconstruction (coronal section on the left and sagittal section on the right) of nectin-1 expression in the developmental cortical wall. **B:** Magnification of the marginal zone and cortical plate. Nectin-1 immunoreactivity was pronounced in the upper tiers of the cortical plate just below the marginal zone. The radially oriented apical dendrites of nectin-1–positive neurons indicated a predominant pyramidal phenotype in layers II/III. **C:** Magnification of the Intermediate zone. Nectin-1 was observed in the cell somata and processes of immature neurons traversing across the corpus callosum to higher cortical destinations, and in the horizontal fiber tracts characteristic of the tangential dispersion of the migratory cells within the intermediate zone. **D:** Magnification of the ventricular zone. Nectin-1 was also detected in a subset of cells in the ventricular zone with characteristics of radial glia-like cells. CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone. Scale bars denote 50 μm, and 20 μm in the insert.

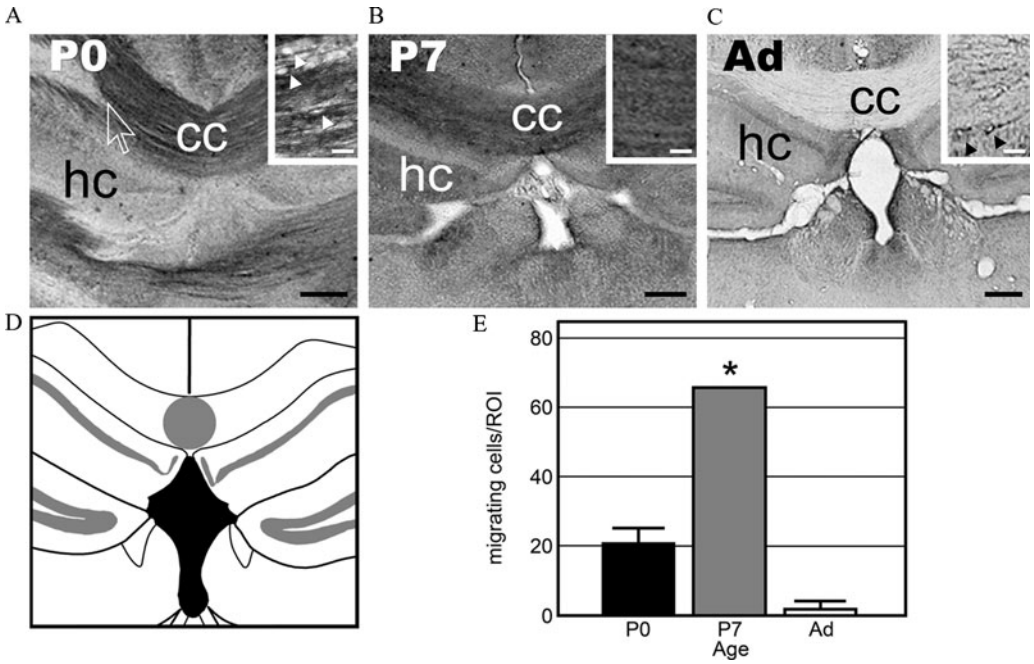

Figure 4 Developmental changes in nectin-1 distribution in the interhemispheric connections. **A:** Coronal section of the brain of newborn mouse (P0). Massive amounts of nectin-1 were detected in callosal bundles and migrating neurons. (*Insert*) Magnified callosal fibers: Many fusiform, spindle-shaped cells displayed a very thin perikaryon with long leading processes all along the callosal fibers (*white arrowheads*). **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. The adult corpus callosum mostly negative for nectin-1 protein expression. (*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. The scale bar denotes 200 μm, and in the insert 20 μm. **E:** Histograms illustrating the age-related numbers (±SEM) of nectin-1–positive migrating cells in the ROI. *Only estimated data; cc, corpus callosum; hc, hippocampus.

Nectin-1 immunoreactivity was prominent in the upper tiers of the cortical plate just below the marginal zone. The radially oriented apical dendrites of the nectin-1–positive neurons indicated a predominant pyramidal phenotype (Figure 3B).

In the intermediate zone, nectin-1 was also observed in the cell somata and processes, and in the horizontal fiber tracts characteristic of the tangential dispersion of migratory cells en route to the cortical plate (Figure 3C).

In the ventricular zone, nectin-1 was detected in a subset of cells with large oval somata, lying close to the ventricular surface. The nectin-1 immunoreactivity was preferentially concentrated at the ventricular pole of the cytoplasm and in the endfeet lining the ventricle (Figure 3D). The position and shape of these nectin-1–positive cells were characteristic of radial glia-like cells, involved in the guidance of neuronal migration.

Nectin-1 is involved in the 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 herpes encephalitis. 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 (Figure 4A, E). Numerous fusiform, spindle-shaped cells displayed a very thin perikaryon with long leading processes all along the callosal fibers (insert in Figure 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 the commissura hippocampi. 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 (Figure 4B). The whole corpus callosum was homogeneous for nectin-1 staining, and the fasciculation of transcallosal axonbundles could no longer distinguished. The nectin-1 signal labeled the callosal area so densely that we could only estimate the number of migrating cells (Figure 4B insert, E). In the adult corpus callosum, however, only faint to no 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 (Figure 4E). Nectin-1 appears to be expressed in the CC of developing mouse brain at stages when HSE shows

bilateral dissemination, whereas it is absent in adult CC when HSE is mostly unilateral.

Discussion

The investigations presented in this report document the spatiotemporal changes in distribution of the primary HSV-1 and HSV-2 neural entry receptor nectin-1 in CNS structures involved in cognitive functioning. This study also provides further insight into the developmental dynamics of the periventricular neurogenic system of the murine brain. This is a particularly relevant finding, because HSV is recognized as a basic factor in the pathogenesis of mental illnesses with increased levels of cognitive impairment (Dickerson *et al*, 2003, 2004; Itzhaki *et al*, 1997).

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

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 (Whitley and Kimberlin, 1999). It has also been suggested that infection of the temporal and frontal cortex results from reactivating virus traveling form trigeminal ganglia, the main site of HSV-1 latency (Baringer and Pisani, 1994; Davis and Johnson, 1979). 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. Damasio and Van Hoesen 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 (Damasio and Van Hoesen, 1985). 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. 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 the 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 the brain development.

With age, there is also a change of frequency of HSE caused by HSV-1 and HSV-2. 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 (Montgomery *et al*, 1996), and HSV-2 might have another unidentified receptor (Zago and Spear, 2003). 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 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.

What does the immunolocalization of nectin-1 tell us about the structural development of the 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 (Figure 5). 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 (Hatten, 1999). In all of these areas, except the olfactory bulb, postmitotic neurons migrate radially away from the germinal zones lining the ventricles, whereas in the olfactory bulb tangential chain-migration occurs (Hatten, 1999; Marin and Rubenstein, 2003). Although well-documented (Hatten, 1999; Marin and Rubenstein, 2003; Parnavelas, 2000), the interaction of the radial and tangential migratory systems during brain development is still unclear. The present study suggests that nectin-1 is involved in both migratory processes. The distribution of nectin-1 in the developing brain suggests an

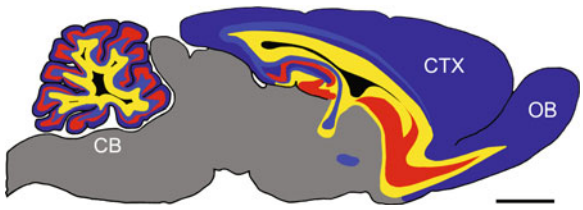

Figure 5 The developmental subunits of murine brain as revealed by nectin-1 immunohistochemistry. Deep blue indicates the main brain areas where the radial migration of neurons occurs, light blue the interhemispherical migratory area of the developing brain, yellow the overall distribution of the neurogenic SVZ, and red the areas remaining plastic during adulthood. CB, cerebellum; CTX, cortex; OB, olfactory bulb. The scale bar on the schematic figure denotes 1 mm.

important role of the intermediate zone in organizing the tangentoradial transition. A similar inference was drawn by Okabe *et al* (2004) 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 the nectin-1 positive fibers guide commissural fiber tracts migrating through the midline in the early stages of perinatal brain development. Recently, Valyi-Nagy *et al* analyzed nectin-1 distribution in the human brain (Valyi-Nagy *et al*, 2005) and in fetal samples. They also found that nectin-1 staining was significantly associated with neurons with migratory phenotypes (personal communication).

Herpes nectin-1—cognitive deficit?

HSV is recognized as one of the factors in the pathogenesis of mental illnesses with increased levels of cognitive impairment. Individuals with schizophrenia, bipolar affective disorder, or Alzheimer's disease who have serum antibodies to HSV-1 exhibit greater levels and an earlier onset of impairment (Dickerson *et al*, 2003, 2004; Itzhaki *et al*, 1997).

Nectin-1α undergoes intramembrane proteolytic processing analogous to that of the amyloid precursor protein in Alzheimer's disease. This enzymatic cleavage is mediated by a presenilin-dependent γ-secretase-like activity (Kim *et al*, 2002). Presenilin 1 and 2 (PS1 and PS2) are polytopic membrane proteins that are mutated in the majority of pedigrees with early-onset familial Alzheimer's disease (Price and Sisodia, 1998). Loss of PS1 and PS2 functions perturbs both the radial and the tangential migration in cerebral cortex (Feng *et al*, 2004; Louvi *et al*, 2004). Moreover, the loss of both PSs results in a massive shrinkage in cortical thickness and reduction of both the transcallosal bundles and the hippocampal molecular layer (Feng *et al*, 2004; Louvi *et al*, 2004). In view of these findings, our observations on expression of nectin-1 in these affected areas indicate a possible functional convergence between nectin-1 and presenillins in a common pathway active in brain morphogenesis.

A growing amount of evidence supports the hypothesis that schizophrenia and Alzheimer's disease include a misconnectivity and disconnectivity syndrome, respectively (Delbeuck *et al*, 2003; Harrison, 1999). Indeed, HSV infection affects the natural adhesive function of nectin-1. During HSV infection, nectin-1 localization at the cellular junction was dramatically altered in a manner dependent on gD expression. Newly synthesized gD can substitutes for nectin-1 of infected cells at homotypic junctions with noninfected cells (Krummenacher *et al*, 2003). In addition, a soluble form of gD is able to dissociate nectin-1–mediated cell contacts (Krummenacher *et al*, 2003). Therefore one can envisage that gD expression during HSE might lead to misconnection of developing *trans*-callosal and temporolimbic pathways in neonates as well as disruption of these preexisting pathways in adults.

Nectin-1 distribution correlates with the long-lasting microglial activation pattern in HSE

Microglia and macrophages are thought to play an important role in the immune response in viral encephalitis (Dorries, 2001). During the course of acute HSE, macrophage infiltration and microglia reaction were detected from 24 h after the initial detection of HSV-1 antigen in the murine brain (Esiri *et al*, 1995). In the case of HSE, the projecting axonal pathways along which microglial activation occurs consist of the large association bundles of the limbic system (Cagnin *et al*, 2001). Interestingly these sites show high levels of expression of nectin-1. It needs to be determined whether nectin-1 is directly involved in recruitment or activation of microglial cells in an analogy to the role of nectin-2 and nectin-like-5 in natural killer (NK) cells stimulation. It is also possible that nectin-1 in these specific areas of the brain may be involved in continued infiltration and activation of macrophages several weeks after virus antigens have been cleared (Cagnin *et al*, 2001; Esiri *et al*, 1995).

As our results have demonstrated, nectin-1 is highly expressed in specific subsets of neurons of the developing murine brain, and this receptor system may play a critical role in spread of HSV as well as in the extent and severity of herpes simplex encephalitis.

Neonatal HSE can have two distinct presentations resulting from the route of infection: a diffuse encephalitis results from generalized viremia and a more unitemporal occurrence is observed when HSV reach the CNS through neuronal spread. In any case the presence of a receptor is required for HSV infection or spread. The route of infection and the distribution of receptor in the brain appear to be equally important to determine the pathogenesis and severity of HSE.

The localization of nectin-1, the function of which is altered by HSV gD binding during HSE, supports a model in which changes in distribution of the synaptic cell adhesion molecule nectin-1 affects the pathogenesis of HSE and its sequelae.

In particular, the psychoimmunobiological features of a cognitive deficit post HSE might result from specific alterations of pathways relying on nectin-1 contacts due to gD binding. This study suggests an important role in pathways involving nectin-1, which disruption might lead to the emergence of mental disorders with a rapid cognitive decline.

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.

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 *et al* (1999) and shows reactivity against mouse and rat neurons (Inagaki *et al*, 2003), synaptosome (Honda *et al*, 2006; Mizoguchi *et al*, 2002), and pigment and nonpigment epithelia (Inagaki *et al*, 2005). The rabbit polyclonal serum R166 raised against human nectin-1 ectodomain shows reactivity against nectin-1 in human and rat neurons (Richart *et al*, 2003; Simpson *et al*, 2005) and mouse epithelium (Shukla *et al*, 2000). The secondary antibodies used and their dilutions were biotinylated anti-rat IgG (H+L), mouse adsorbed, made in rabbit (Vector Labs), 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 (Figure 1C, E). The slides were processed digitally (Olympus BX51, DP70, and Olympus DP Manager).

Quantification of nectin-1–positive migrating cells in the corpus callosum. The number of nectin–1 positive cells in the corpus callosum of P0, P7, and adult mice was calculated as follows. For each group, fusiform, spindle-shaped cells exhibiting a very thin perikaryon with leading and lagging processes were counted in the region of interest (Figure 4D) in five consecutive sections from five animals. We were able to determine the exact cell counts in the newborn and adult mice. However, in the P7 animals, the nectin-1 immunosignal was so intense that the number of migrating cells could only be estimated. The numerical values for P0, P7, and adult mice were graphed.

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