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Intranasal delivery of cells to the brain

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Abstract

The safety and efficacy of cell-based therapies for neurodegenerative diseases depends on the mode of cell administration. We hypothesized that intranasally administered cells could bypass the blood-brain barrier by migrating from the nasal mucosa through the cribriform plate along the olfactory neural pathway into the brain and cerebrospinal fluid (CSF). This would minimize or eliminate the distribution of cellular grafts to peripheral organs and will help to dispense with neurosurgical cell implantation. Here we demonstrate transnasal delivery of cells to the brain following intranasal application of fluorescently labeled rat mesenchymal stem cells (MSC) or human glioma cells to naive mice and rats. After cells crossed the cribriform plate, two migration routes were identified: (1) migration into the olfactory bulb and to other parts of the brain; (2) entry into the CSF with movement along the surface of the cortex followed by entrance into the brain parenchyma. The delivery of cells was enhanced by hyaluronidase treatment applied intranasally 30 min prior to the application of cells. Intranasal delivery provides a new non-invasive method for cell delivery to the CNS.

Keywords: Intranasal delivery; Mesenchymal stem cells; Glioma cells; Hyaluronidase; Olfactory route; Trigeminal route; CD45; Stem cell transplantation

Introduction

The success of cell-based therapy for neurodegenerative disorders depends on the therapeutic properties of the cell type, on the method and safety of administration, on the amount of cells delivered to the site of injury and finally on the avoidance of excessive incorporation of the therapeutic cells into other organs and systems. Transplantation of therapeutic cells into the brain is one of the most frequently used invasive approaches to promote recovery of the CNS from injury or disease. However, methodologically, transplantation may raise problems not only because of graft rejection as a result

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of an immunological response to the transplants (Kozłowska et al., 2007; Sinden et al., 1992; Li et al., 2008) but also due to the mechanical tissue damage from this traumatic and expensive surgical procedure.

The low efficacy of intrathecal application of human bone marrow-derived mesenchymal stem cells (MSC) in amyotrophic lateral sclerosis was attributed primarily to the route of application (Habisch et al., 2007). Disease progression can also reduce the likelihood of graft survival and maintenance after transplantation: grafted neural progenitor cells survived in animals with mild but not in those with severe traumatic brain injury (Shindo et al., 2006).

Insufficient graft survival was shown in animal models and patients with Parkinson's disease (Björklund, 2005; Helt et al., 2001) and even in the brains of normal rodents (Coyne et al., 2006, 2007). The above studies call for the development of non-invasive methods of targeted cell delivery which favor cell homing towards the CNS and reduce the side effects associated with the accumulation and activities of therapeutic cells outside of the brain.

We hypothesized that intranasally administered cells could bypass the blood-brain barrier by migrating from the nasal mucosa through the cribriform plate along the olfactory neural pathway into the brain and cerebrospinal fluid (CSF). This would minimize or eliminate the distribution of cellular grafts to peripheral organs and obviate the need for neurosurgical cell implantation. This idea was tested by intranasal (IN) administration of mesenchymal stem cells to adult mice and administration of glioma cells to young rats. We also hypothesized that IN hyaluronidase treatment, which has been shown to facilitate transnasal invasion of the bloodstream and infection of the CNS by pneumococci (Zwijnenburg et al., 2001), would improve the delivery of MSC to the brain.

Here we demonstrate for the first time non-invasive IN delivery of mesenchymal stem cells and glioma cells to the brains of rodents and the enhancement of cell delivery with hyaluronidase. This biological pathway of cell migration from the nasal mucosa to the brain thus provides an opportunity for the development of cell delivery methods for therapeutic and experimental use in treating neurodegenerative disorders and creating brain tumor models.

Materials and methods

Isolation and cultivation of bone marrow MSC

MSC were isolated from the mononuclear cell fraction of pooled bone marrow (BM) from 10 Dark Agouti rats (female, age: 1 month) after plastic adherence and culture (37 °C, 5% humidified CO_2) in α-MEM (Cambrex Bio Science, Verviers, Belgium), containing desoxyribonucleotides, ribonucleotides, ultra glutamine 1, 100 I.U./ml penicillin (Cambrex Bio Science), 100 μg/ml streptomycin (Cambrex Bio Science) and 10% heat-inactivated fetal calf serum (FCS, Cambrex Bio Science). To determine the stem cell plasticity, tri-lineage in vitro differentiation was performed as described previously (Ji et al., 2004) and demonstrated by specific staining with oil red O (adipogenesis), alkaline phosphatase (osteogenesis) and safranin O (chondrogenesis). FACS analysis was performed using a FACScan (BD Biosciences, San Jose, CA, USA) with BD CellQuestPro software and the PE-conjugated antibodies anti-rat-CD9, -CD11b/c, -CD29, -CD31, -CD34, -CD39, -CD44, -CD45, -CD73, -CD90, -CD106, -CD117, -CD133, -CD143, -CD166, -CD200, -SSEA-1 (BD Biosciences; Santa Cruz Biotechnology, Santa Cruz, CA, USA, Serotec, Raleigh, NC, USA; Biolegend, San Diego, CA, USA).

Fluorescent labeling of cells

One day prior to labeling, the culture medium was removed and the cells (MSC or glioma cells) were washed twice with sterile PBS. Carboxyfluorescein diacetate (1 μM; CFDA, V12883, Invitrogen, Karlsruhe, Germany) or 1 μM Hoechst 33342 (Sigma, Taufkirchen, Germany) both dissolved in sterile PBS was added and the culture was incubated for 30 minutes at 37 °C in an incubator. Cells were washed with PBS twice and incubated in Dulbecco's Modified Eagle's Medium/10% (v/v) FCS overnight. On the day of application, cells were washed again with PBS and harvested for IN application (3×10^5 cells in 24 μl sterile PBS per application).

Intranasal application (INA) of cells

All animal experiments were approved by the local institutional and governmental committee on animal care. All animals used in this study were purchased from Charles River (Sulzfeld, Germany). C57BL/6 mice received rat MSC, whereas 10-day-old Wistar rats received human PhiYellow-T406 glioma cells (Marinpharm, Berlin, Germany). The animals were divided in 3 groups ($n = 5$ in each group), the first group received only the cells (3×10^5 cells/animal), the second group received 100 U hyaluronidase (Sigma) dissolved in sterile PBS 30 minutes prior to the application of cells, the third group was treated with vehicle (PBS). For INA, a total volume of 24 μl of cell suspension or hyaluronidase solution or vehicle was used. The mice were held with a hand grip that allowed the animals to recline on their backs while immobilizing the skull, and the nose drop containing the substance/cell suspension was carefully placed on one nostril allowing it to be snorted essentially

as previously described for intranasal administration of drugs to mice by Hanson et al. (2004). Mice received twice for each nostril alternate applications (left-right) of gradually applied 6-µl drops containing cell suspension/hyaluronidase or PBS (the second set of right-left applications was performed 2 minutes after the first set). Animals were sacrificed under ketamine anesthesia (75 mg/kg, i.p.) 1 h after the application of cells.

To also investigate whether tumor cells may be delivered to the brain after INA, human T406 glioma cells stably transfected with a construct encoding Phi-Yellow (Marinpharm, Berlin, Germany) were intranasally applied to 10-day-old Wistar rats ($n = 5$). Prior to INA, the cells were additionally labeled with 1 µM CFDA, endowing them with strong fluorescence and enhancing their detectability in the brain. One hour after INA of 3×10^5 cells, the animals were sacrificed under ketamine anesthesia (75 mg/kg i. p.), and the whole heads were frozen. Tissue sections (20 µm) were prepared from the whole heads, including the scalp of animals, and screened for the presence of CFDA-positive T406 glioma cells by fluorescence microscopy.

Preparation of brain sections

Sagittal or horizontal cryosections (20 µm in thickness) were prepared from the whole heads or brains of rats and mice. Brain sections of animals treated with HD33342-labeled MSC (blue fluorescence) were coated with Vectashield mounting medium (Vector Laboratories Burlingame, CA) containing propidium iodide (PI, red) in order to discriminate between MSC and nuclei of the host brain. Brain sections of animals treated with CFDA-labeled MSC were mounted with Vectashield mounting medium containing 4′,6 diamidino-2-phenylindole (DAPI, Vector Laboratories Burlingame, CA). All brain sections were analyzed by fluorescence microscopy using an Olympus BX51 microscope and the ''AnalySIS'' software (Soft Imaging System GmbH, Leinfelden-Echterdingen, Germany). HD33342-labeled MSC were counted in the sagittal sections from the area of ±2 mm from bregma from left to right.

Immunohistochemical analyses of CD45

Brain sagittal sections of treated mice (C57BL/6, 6 months old) were screened for CFDA-labeled MSC (1 h after application of MSC). Selected slices in which MSC had been identified were stained for CD45. Sections were fixed with methanol at −20 °C, washed and incubated with CD45 mouse monoclonal antibody (dilution 1:100; BD Biosciences Pharmingen, San Diego, CA) for 2 h at room temperature (RT).

Sections were washed with PBS and further incubated with Cy3-conjugated goat anti-mouse IgGs diluted 1:500 (Dianova, Hamburg, Germany) for 1 h at RT. To obtain negative controls, the samples from the neighboring slices stained for CD45 were incubated with secondary antibody Cy3-conjugated goat anti-mouse IgGs only. Thereafter, samples were washed with 0.1% Triton X-100 (Sigma) in PBS, coated with Vectashield mounting medium (Vector Laboratories Burlingame, CA) containing DAPI and assessed by fluorescence microscopy.

Statistical analyses

Statistical analyses were performed using the two-tailed *t*-test and single comparison of respective groups. $p < 0.05$ was considered significant ($^*p = 0.02$–0.03). The data are presented as mean ± SEM.

Results

The efficacy of IN delivery of cells to the brain was assessed by IN application (INA) of fluorescently labeled rat MSC to adult C57BL/6 mice. Delivery of MSC to the brain was investigated in two separate experimental sets with two different fluorescent dyes: Hoechst 33342 (HD33342) and carboxyfluorescein diacetate (CFDA). We compared brain sections of hyaluronidase-treated and naive animals one hour after INA of MSC. HD33342-labeled cells were detected in the brain sections of animals following INA of MSC (without hyaluronidase) throughout the layers of the olfactory bulb (OB) (Fig. 1A, arrows), thalamus (Fig. 1C, arrows), hippocampus (Fig. 1C, arrowheads), and cerebral cortex (Fig. 1E, arrows). Hyaluronidase treatment prior to the INA of cells resulted in considerably enhanced delivery of MSC to the OB (Fig. 1B, arrows), whereas incorporation of labeled cells into the hippocampus (Fig. 1D, arrowheads), thalamus (Fig. 1D, arrows) and cortex (Fig. 1F, arrows) was only slightly increased. Following INA, only singular MSC were seen in the cerebellum of hyaluronidase-treated animals (data not shown) or in animals not treated with the enzyme (Fig. 2A, arrows).

One hour after INA of CFDA-labeled MSC, numerous cells were found in the subarachnoid space (Fig. 2B, arrowheads) in close vicinity to MSC which had already reached the upper layer of the cortex (Fig. 2B, arrow). Some of these cells developed processes (Fig. 2B, arrow), suggesting ongoing in vivo differentiation of MSC. The amount of CFDA-labeled cells found in the OB was much higher than in all other areas of the brain (Fig. 2D). A large amount of the IN-applied CFDA-labeled MSC remained in the upper nasal cavity

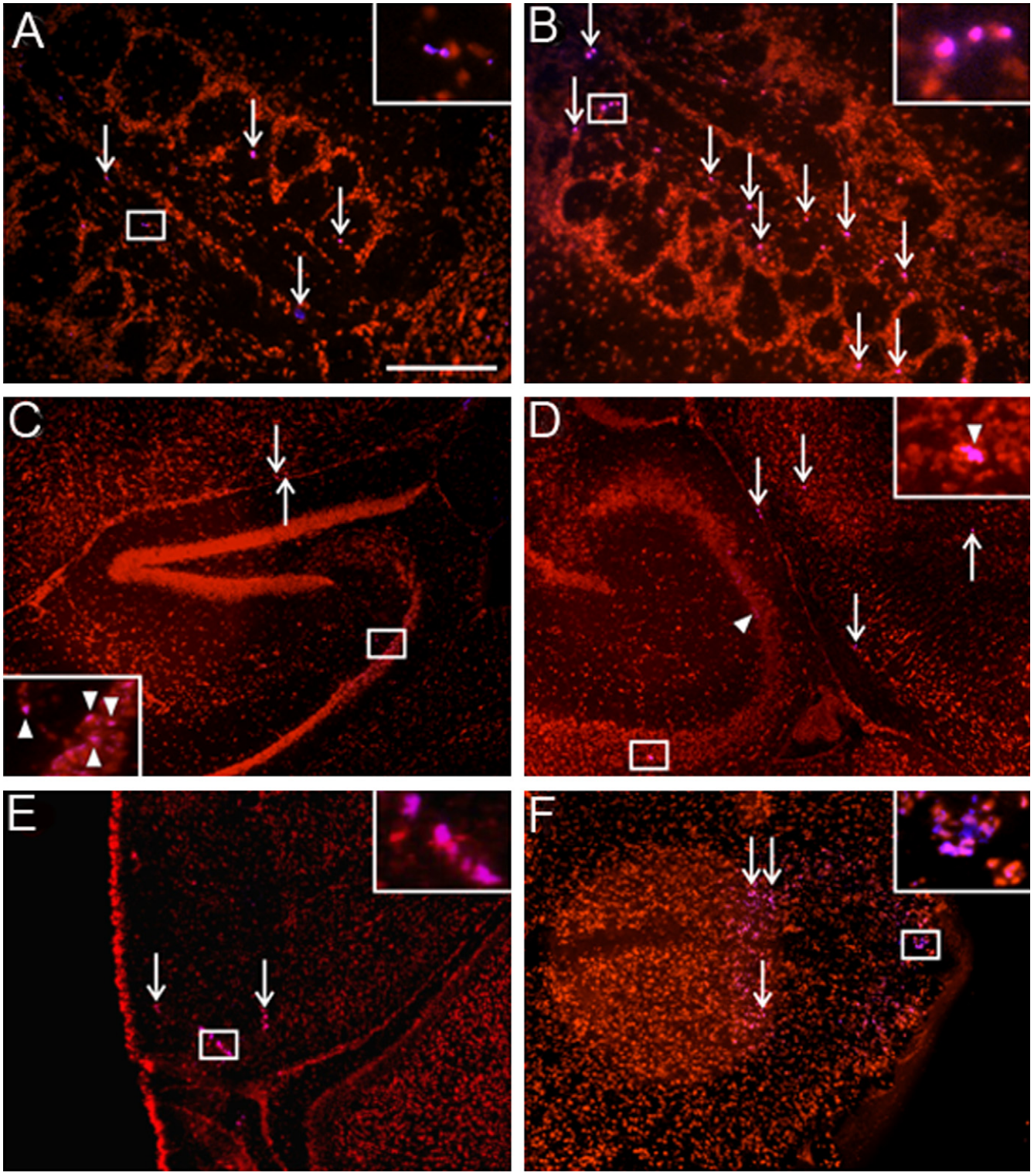

Fig. 1. Brain sections of 6-month-old C57BL/6 mice 1 h after INA of Hoechst 33342 (HD33342; blue)-labeled rat MSC (3×10^5 cells/animal). Animals either received IN-applied cells only (A, C, E) or were pretreated with 100 U hyaluronidase 30 min prior to application of cells (B, D, F). The brain sections were treated with propidium iodide (PI, red) to visualize the nuclei of both host cells and IN-applied HD33342-labeled MSC. The MSC (pink-colored cells in merged micrographs) were found in the area of the olfactory bulb (A, B, arrows), hippocampus (C, D, arrowheads), thalamus (C, D, arrows), and cortex (E, F, arrows). The pink color of MSC resulting from the superimposition of the red PI and the blue HD33342 color is better seen under higher magnification (A-F, inserts). The highest number of IN-delivered cells was seen in the OB of hyaluronidase-treated animals (B). Bar: 500 μm (A-F).

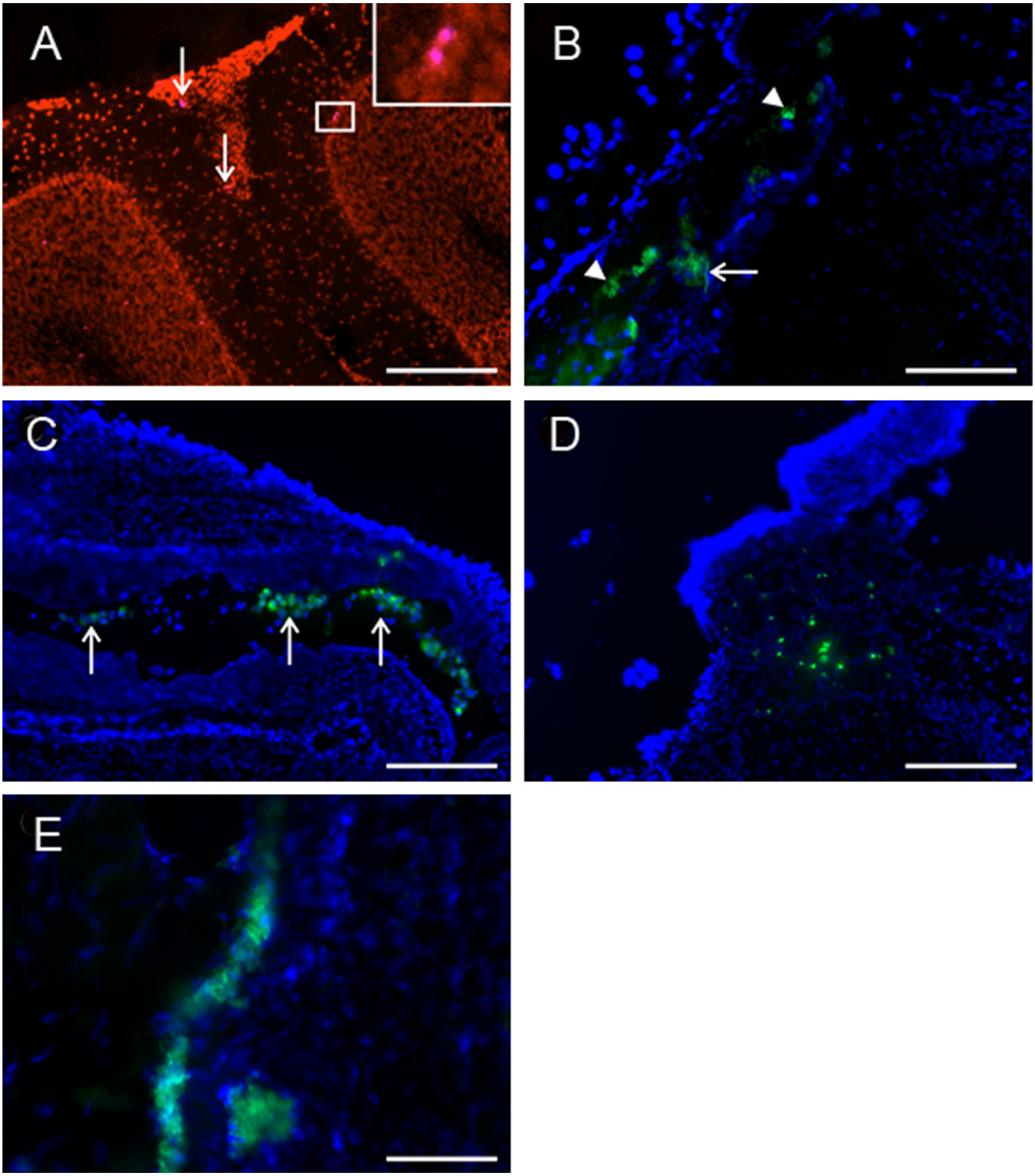

Fig. 2. Brain sections of 6-month-old C57BL/6 mice after INA of Hoechst 33342 (HD33342; blue)- or CFDA (green)-labeled rat MSC (3×10^5 cells/animal). The slices were stained with propidium iodide (PI, red) or DAPI (blue) to demonstrate the nuclei of both, host cells and IN-applied MSC. In PI-treated brain sections, the HD33342-labeled MSC could be distinguished by their pink color (A). Small and large inserts in (A) show the same group of cells under different magnifications. In DAPI-treated brain sections (B-E), the IN-delivered MSC could be distinguished by green fluorescence due to CFDA. The IN-delivered MSC were found in different areas of the brain including cerebellum (A), subarachnoid space (B, arrowheads) and outer layer of cortex (B, arrow), upper nasal cavity (C), throughout the layers of OB (D) and cortex (E). Bars: 500 μm (A, C, D); 100 μm (B, E).

(Fig. 2C, arrows). A stepwise migration of cells from the surface into the deeper layers is suggested by the discontinuous chain of cells in the cortex (Fig. 2E). To estimate the amount of cells arriving in the brain via the IN route and to assess the effect of hyaluronidase on the efficacy of INA, we quantified MSC in the brains of C57BL/6 mice. HD33342-labeled cells were counted throughout the sagittal sections in an area of ± 2 mm from bregma. Outside of this area, only a negligible number of labeled cells was found. Of 3×10^5 IN-applied cells, 584 ± 152 arrived in the OB, 227 ± 47 in the cortex, and 210 ± 60 in the remaining areas of the brain (Fig. 3). Hyaluronidase-treated animals contained significantly more CFDA-labeled cells in OBs (OB in Fig. 3) and total brain area under investigation (H total in Fig. 3) than animals without hyaluronidase treatment. This effect of hyaluronidase was less pronounced in the cortex (cort in Fig. 3).

In order to assess whether MSC were phagocytosed by immunocompetent cells after INA, brain sections of adult mice containing CFDA-positive MSC were stained for CD45 (Fig. 4A–D), a marker protein of blood-derived cells and activated microglia (Sedgwick et al., 1991) known to show phagocytic activity. The CFDA label of MSC (Fig. 4A, arrow) was detected apart from CD45-positive cells (Fig. 4A, B, arrowhead) in the brain cortex and on the way through the cribriform plate to the OB (Fig. 4C, D, arrow), but occasionally coincided with CD45-positive cells. Interestingly, CD45-positive cells are capable of crossing the cribriform plate, as shown in Fig. 4C and D (arrowheads). Analyses of the brains of 6-month-old C57BL/6 mice after INA of CFDA-labeled rat MSC revealed that some of the exogenously applied cells can be destroyed by blood-derived monocytes or microglia, as evidenced by co-localization of CD45 and CFDA in few cells detected in the striatum (Fig. 5A, B).

To investigate whether tumor cells also may be delivered to the brain after INA, we applied human PhiYellow/CFDA-labeled T406 glioma cells to young (10-day-old) Wistar rats. The glioma cells were detected in the process of migration from the nasal mucosa (Fig. 5C, arrowheads) through the cribriform plate (asterisk) to different areas of the brain such as the OB (Fig. 5D, arrow), frontal cortex (Fig. 5E, arrow) and hippocampus (Fig. 5F, arrow) 1 h after application.

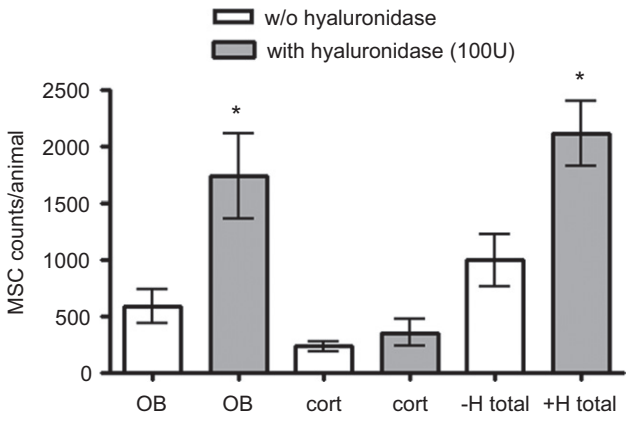

Fig. 3. Quantification of rat MSC in the brain sections of C57BL/6 mice. After INA of Hoechst 33342-labeled rat MSC (3×10^5 cells/animal) the quantification of rat MSC in the olfactory bulb (OB), cortex (cort), and total number ($\pm$H total) of MSC found throughout all brain areas investigated was performed using serial brain sections of naïve rats (white columns) and that of hyaluronidase pre-treated rats (grey columns); $n = 5$ per group. The data are presented as mean $\pm$ SEM. Statistical analyses were performed using two-tailed *t*-test. $p < 0.05$ was considered significant ($^*p = 0.02$ in comparison of OB with and without hyaluronidase; $^*p = 0.04$ in comparison of $\pm$H total).

Discussion

In the present study, the IN delivery of mesenchymal stem cells and glioma cells to the brain demonstrates that cells applied intranasally can migrate to the intact brain through the cribriform plate along the olfactory neural pathway and possibly along other routes of migration. The fact that IN-applied cells can travel though the cribriform plate is consistent with several reports showing that during prenatal development, the neurons secreting gonadotropin-releasing hormone migrate from the nasal compartment as the site of their origin via the cribriform plate into the forebrain (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Wray, 2001). The existence of a large number of the IN-applied CFDA-labeled MSC in the upper nasal cavity indicates that cell migration from the nasal mucosa through the cribriform plate into the brain could possibly proceed for several hours and perhaps even days. The delivery of human adipose-derived MSC to the brain after intravenous administration was previously shown in several xenotransplantation models using immunodeficient mice (Meyerrose et al., 2007). However, the authors reported high delivery of these cells to the peripheral organs: besides skeletal tissue and heart, the brain was the organ with the lowest amount of delivered cells in comparison with delivery to lung, liver, kidney and spleen. Intranasal application of cells provides an alternative method of delivery to the brain that may reduce systemic exposure. However, further investigation is needed to elucidate the possible distribution of IN-applied cells outside of the brain and their fate within the brain after extended periods of time.

Intranasal pre-treatment with hyaluronidase improved the delivery of MSC to the brain. Zwijnenburg et al. (2001) showed that hyaluronidase facilitates the passage of pneumococci from the nasal cavity into the

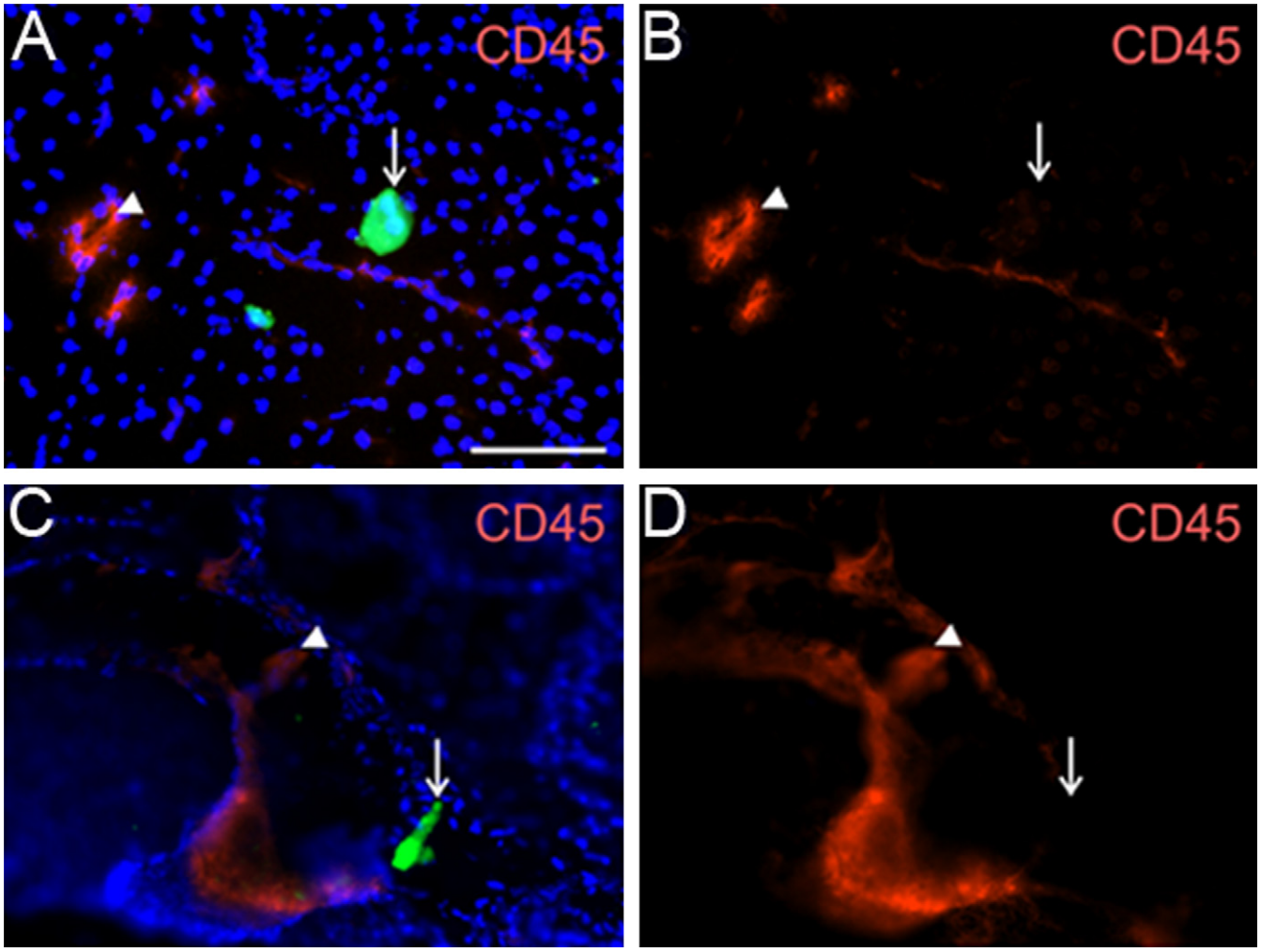

Fig. 4. Expression of CD45 in the brain cortex of 6-month-old C57BL/6 mice after INA of CFDA (green)-labeled rat MSC (3×10^5 cells/animal). (A) CD45-positive (red) cells indicated by the arrowhead in the merged micrograph lack CFDA (green). (B) CFDA-containing MSC are negative for CD45 (A, B, arrows). (C) CD45-positive cells (C, D, arrowhead) and CFDA-positive MSC (C, arrow) crossing the cribriform plate. (D) MSC are negative for CD45 (D, arrow); Bar: 100 μm (A-D).

blood stream and the subsequent induction of pneumococcal meninigitis, while IN inoculation of *Streptococcus pneumoniae* without hyaluronidase treatment does not lead to systemic infection. It was suggested that hyaluronidase facilitates the invasion by loosening the barrier function of the nasopharyngeal mucosa.

Immunolabeling of brain sections containing CFDA-positive MSC with CD45 antibodies revealed that the majority of MSC was not phagocytosed by immunocompetent cells after INA. MSC delivered into the brain likely escape destruction by blood-derived and brain-specific immunogenic cells because they are not inherently immunogenic and possess strong immunomodulatory capacities reflected by their ability to suppress the activation of natural killer cells and T-cells (Krampera et al., 2003; Sotiropoulou et al., 2006).

Here we also show rapid entry of glioma cells into the brains of young rats following INA. The fact that intranasally applied human glioma cells can migrate across the cribriform plate into many brain areas including the olfactory bulb, frontal cortex and hippocampal area raises the possibility that primary tumors located outside of the brain may be able to metastasize to the brain along this same pathway, perhaps first reaching the olfactory epithelium through its intimate connection with the lymphatic system and deep cervical lymph nodes.

The fact that the detectable amount of IN-applied cells is rapidly delivered to the CNS (after 1 h) suggests the possible transport of the cells through the perivascular spaces which previously have been proposed to serve as a conduit for the rapid distribution of molecules within the CNS (Gregory et al., 1985; Rennels et al., 1985, 1990; Cserr and Ostrach, 1974; Hadaczek et al., 2006) and for the rapid delivery of drugs from the nose to the brain (Thorne et al., 2004). For example, viral

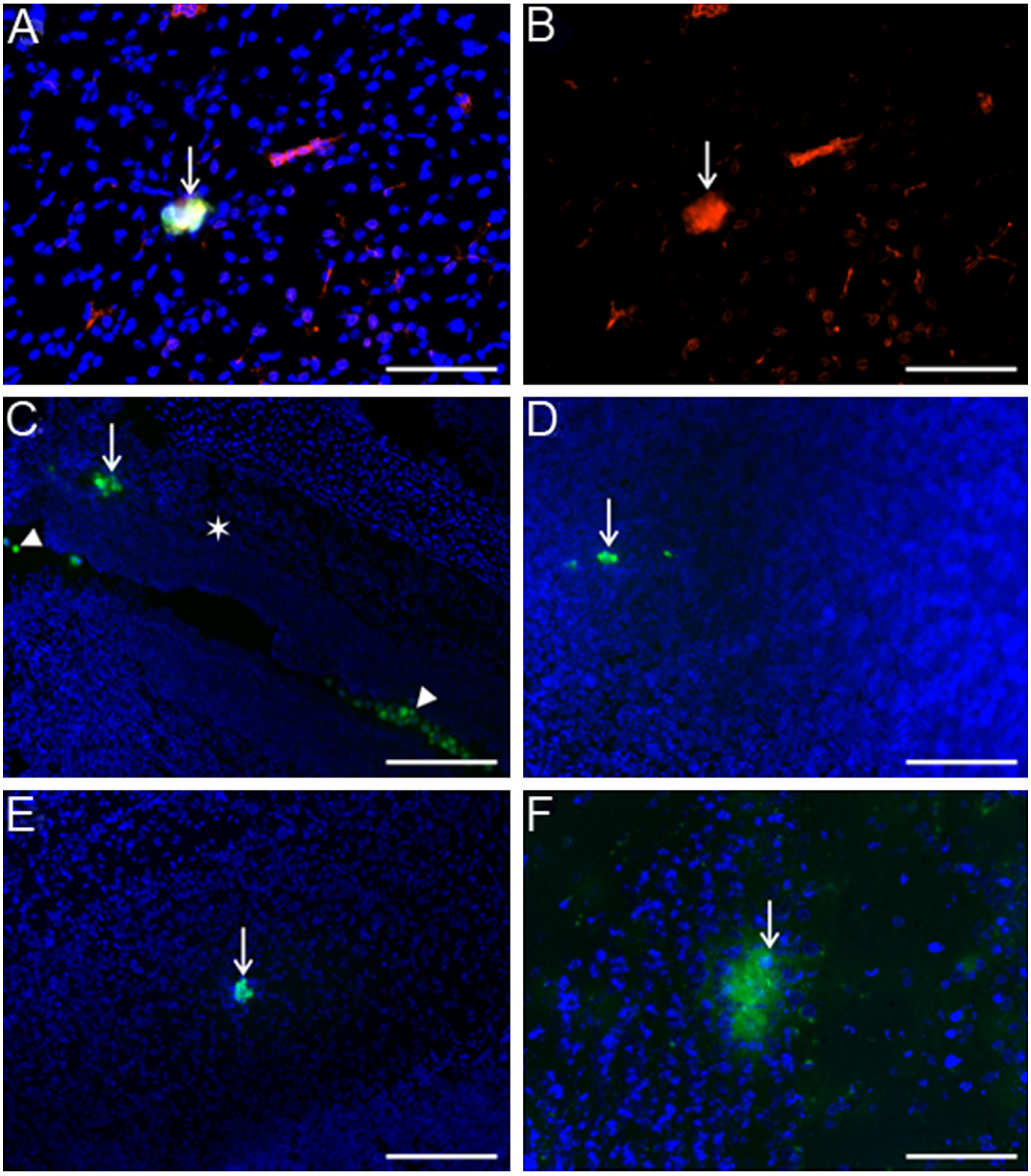

Fig. 5. Detection of CFDA-labeled MSC in mice and PhiYellow/CFDA-labeled T406 human glioma cells in rats. (A) Staining for CD45 (red) and fluorescent nuclear counterstain with DAPI (blue) of a head section of a 6-month-old mouse after INA of CFDA-labeled (green) rat MSC (3×10^5 cells/animal). Merged micrograph shows the location of CFDA-labeled MSC (green) in the striatum. (B) The presence of CD45 in MSC (arrow) suggests that the IN-applied cells can partially be destroyed by blood-derived monocytes or microglia. (C-F) IN delivery of PhiYellow/CFDA-labeled T406 human glioma cells (green) into 10-day-old Wistar rats. Asterisk in (C) indicates the cribriform plate; Phi-Yellow/CFDA-double-labeled glioma cells were identified in nasal mucosa (C, arrowheads), cribriform plate (C, arrow), olfactory bulb (D, arrow), frontal cortex (E, arrow) and hippocampal area (F, arrow). Bars: 100 μm (A, B, F); 500 μm (C-E).

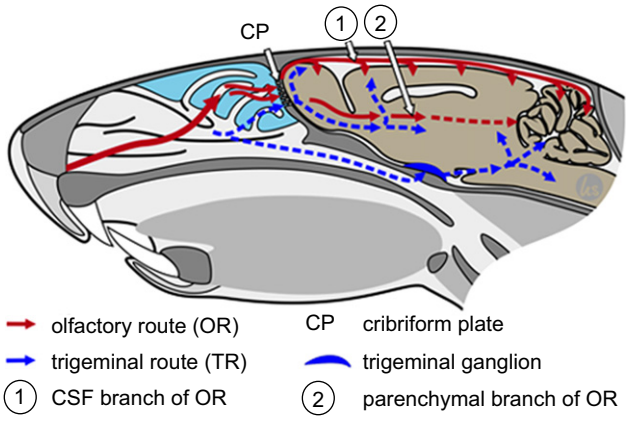

Fig. 6. Schematic drawing of two routes of IN delivery of cells to the brain. After crossing the cribriform plate (CP), the olfactory route (OR, red arrows) divides into two branches: (1) the CSF branch and (2) the parenchymal branch. Solid arrows represent the paths of migration of cells into the brain evidenced in this study, whereas dashed arrows reflect possible hypothetical routes of cell delivery. The hypothetical trigeminal route (TR) consists also of at least two branches one of which crosses the cribriform plate into the parenchyma, where it diverges to the rostral and caudal parts of the brain. The second branch projects from the nasal mucosa to the trigeminal ganglion, where the exogenously applied cells are further distributed to the forebrain, OB and caudal brain areas including the brainstem and the cerebellum.

capsids and macromolecules like albumin or liposomes injected into the striatum in rats were shown to be transported to the globus pallidus within 30 minutes (Hadaczek et al., 2006). The authors of this work demonstrated rapid transport of particles/molecules within the basal ganglia proposing the concept of a "perivascular pump" driven by arterial pulsation. This "perivascular pump" may also be involved in the rapid transport of cells within the brain parenchyma.

The results presented herein provide evidence for at least two branches of the olfactory route (OR) of cell migration from the nasal mucosa into the brain (red solid arrows, Fig. 6). The first branch is the parenchymal route which may partially be associated with the rostral migratory stream (RMS), the known major track by which neural progenitor cells migrate from the subventricular zone of the lateral ventricle to the OB (Lois and Alvarez-Buylla, 1994; Luskin, 1993).

The majority of hematopoietic stem cells injected into the OB have been shown to use the RMS for bi-directional migration (Moore et al., 2005). The second branch of the OR passes through the CSF, which has been proposed to be one of the possible routes of IN-delivered drugs into the brain (Thorne et al., 2004).

Close analysis of the loci of MSC accumulation in the brain and of the literature about the possible routes of cerebral trafficking of compounds and cells suggest two additional potential migratory paths (dashed arrows, Fig. 6): (a) the trigeminal route (TR, Fig. 6) and (b) the perivascular route (not shown) which may both serve as side-tracks for cell movement from the nasal mucosa into the brain. The importance of the perivascular route as a conduit of cell trafficking is further emphasized by a recent study demonstrating the migration of OB neuroblasts along blood vessels (Bovetti et al., 2007).

In this study, the direct IN delivery of cells to the brain has been demonstrated for the first time. The potential value of this principally new approach for the therapy of neurodegenerative disorders and for the generation of disease models was shown by the employment of MSC and of glioma cells, respectively. The successful delivery of cells to the CNS following INA opens new avenues for the use of this method as a non-invasive alternative to the current traumatic surgical procedure of transplantation. The IN delivery method provides the option of chronic treatment which may enhance the number of delivered cells in order to achieve therapeutic benefit. However, successful delivery of cells does not, of course, assure therapeutic success which is primarily dependent on the therapeutic efficacy of the respective cell type applied. Further studies on various models of chronic and acute neurodegeneration are needed to improve the efficacy and assess the safety of this natural migratory track.

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