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Delayed Transplantation of Human Neural Precursor Cells Improves Outcome from Focal Cerebral Ischemia in Aged Rats

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SUMMARY

Neural precursor cell (NPC) transplantation may have a role in restoring brain function after stroke, but how aging might affect the brain's receptivity to such transplants is unknown. We reported previously that transplantation of human embryonic stem cell (hESC)-derived NPCs together with biomaterial (Matrigel) scaffolding into the brains of young adult Sprague-Dawley rats 3 wks after distal middle cerebral artery occlusion (MCAO) reduced infarct volume, and improved neurobehavioral performance. In this study we compared the effect of NPC and Matrigel transplants in young adult (3-mo-old) and aged (24-mo-old) Fisher 344 rats from the National Institute on Aging's aged rodent colony. Distal MCAO was induced by electrocoagulation and hESC-derived NPCs were transplanted into the infarct cavity 3 wks later. Aged rats developed larger infarcts, but infarct volume and performance on the cylinder and elevated body swing tests, measured 6–8 wks post-transplant, were improved by transplantation. We conclude that advanced age does not preclude a beneficial response to NPC and Matrigel transplantation following experimental stroke.

Keywords

transplant; neural precursor; ischemia; stroke; brain

INTRODUCTION

Transplantation of exogenous neural precursor cells (NPCs) is a potential strategy for restoring brain function after stroke (Bliss *et al.* 2010). In previous studies, NPCs of rodent (Wei *et al.* 2005; Zhu *et al.* 2005; Daadi *et al.* 2009a), nonhuman primate (Hayashi *et al.* 2006) and human (Chu *et al.* 2004; Ishibashi *et al.* 2004; Kelly *et al.* 2004; Chu *et al.* 2005; Daadi *et al.* 2009b; Daadi *et al.* 2010) origin have all been transplanted into ischemic rodent brains, where they have been found to survive and improve histological or functional outcome. Although most potential recipients of human NPC transplants for stroke are elderly and aging may adversely affect stroke outcome (Nakayama *et al.* 1994; Ay *et al.* 2005), little attention has been given to how aging might affect the brain's receptivity to such transplants.

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We and others have found that transplantation of NPCs together with biomaterial scaffolding improves transplant survival and function in the ischemic young rodent brain (Park *et al.* 2002; Bible *et al.* 2009; Jin *et al.* 2010; Zhong *et al.* 2010). In our study (Jin *et al.* 2010), human NPCs derived from human embryonic stem cell (hESC) line BG01 were transplanted with Matrigel scaffolding into young adult (280–310 g) Sprague-Dawley rat brain 3 wks after middle cerebral artery occlusion (MCAO). Five to nine weeks later, rats given NPC-Matrigel transplants showed increased survival of transplanted cells, reduced infarct volume, and improved performance on neurobehavioral tests, compared to rats given vehicle, NPCs, or Matrigel alone.

Here we report the results of studies to determine if the salutary effects of human NPC and Matrigel transplantation on outcome from MCAO extend to aged rodents.

RESULTS

To determine if the benefits of delayed transplantation extend to older rats, who model more closely the age group most susceptible to stroke in humans, the same nestin^{+}/SOX1^{+}/calbindin^{-}/GFAP^{-}/OX4^{-} NPCs used in our previous study (Jin *et al.* 2010), mixed with Matrigel scaffolding, were transplanted 3 wks post-MCAO into the infarct cavity of 3- and 24-mo-old Fisher rats. A different rat strain was used because the NIA aged rodent colony from which we obtained our animals employs Fisher rather than Sprague-Dawley rats. Figure 1 shows that infarct volume 11 wks post-MCAO (8 wks post-transplant) was ~30% greater in 24- than in 3-mo control rats given aCSF injections into the infarct cavity. An age-related increase in infarct size or evolution rate in rodents has been reported in some but not other prior studies (Popa-Wagner *et al.* 2007). In rats who received NPC transplants, infarct volume was reduced—by ~50% ($p<0.05$) in 3-and ~40% ($p<0.05$) in 24-mo-old animals.

The reduction in infarct volume we observed previously in young adult rats given NPC transplants after MCAO was accompanied by improvement in a battery of neurobehavioral tests designed to detect long-term post-ischemic deficits (Jin *et al.* 2010). In this study we also assessed neurobehavioral outcome, using the cylinder test (Schallert *et al.* 2000) and elevated body swing test (EBST) (Borlongan & Sanberg 1995), which detect asymmetries in forelimb use and rotational behavior, respectively. In the cylinder test, 3-mo-old rats showed ~30% preferential use of the unaffected limb 6 wks post-transplant, which had essentially resolved by 8 wks (Figure 2). The preference observed at 6 wks was reduced to ~15% ($p<0.05$) in rats given NPC transplants. By comparison, 24-mo-old rats showed greater (~70%) preferential use of the unaffected limb at 6 wks post-transplant, which decreased only slightly (to ~50%) at 8 wks. NPC transplants reduced the preference for unaffected limb use, to ~30% ($p<0.05$) at 6 wks and ~15% ($p<0.05$) at 8 wks.

In the EBST, 3-mo-old rats showed ~60% preferential rotation away from the ischemic hemisphere, which was similar at 6 and 8 wks post-transplant, and was not reduced by NPC transplants. In contrast, 24-mo rats exhibited greater rotational preference, but this preference was reduced significantly by transplantation, from ~90% to ~70% ($p<0.05$) at 6 wks and from ~80% to ~50% ($p<0.05$) at 8 wks post-transplant. Thus, despite more severe neurobehavioral deficits in 24- than in 3-mo-old rats under control (non-transplanted) conditions, 24-mo rats showed improved performance following NPC transplantation.

To investigate the survival and phenotypic fate of transplanted NPCs, brain sections from 24-mo-old rats who underwent MCAO, NPC transplantation 3 wks later, and bromodeoxyuridine (BrdU) administration followed by euthanasia 8 wks after that, were stained with antibodies against human and cell type-specific marker proteins. Figure 3 illustrates that HuN-immunopositive (transplanted human) cells were abundant at the

transplant site, and that some HuN-positive cells were labeled with BrdU, suggesting that they had divided during the 24-hr period between BrdU administration and euthanasia. Other HuN-positive cells stained for caspase-3 cleavage product, indicating that they were undergoing caspase-dependent cell death.

In our previous study of NPC transplantation in young adult Sprague-Dawley rats, we found that transplanted cells went on to express markers of both neuronal and astroglial lineage (Jin *et al.* 2010). As shown in Figure 4, the same was true for 24-mo-old Fisher rats in the present study. Thus, we observed co-expression of the transplanted human cell marker HuN with three neuronal lineage markers—βIII tubulin, doubklecortin (DCX), and calbindin—and the astroglial marker glila fibrillary acidic protein (GFAP). DCX, which is associated most notably with migrating new neurons, was seen in cells that also exhibited features of migratory morphology.

DISCUSSION

Transplantation of several tissues—including kidney (Saxena *et al.* 2009), heart (Aliabadi *et al.* 2007), liver (Keswani *et al.* 2004), and bone marrow (Jantunen 2006)—can provide therapeutic benefit in elderly patients. The prospect of cell transplantation for stroke is also appealing, but what special problems the aged recipient may present are unknown (Bliss *et al.* 2010). One concern is that the aged brain may be less receptive to the survival, proliferation, and function of transplanted neural cells. This arises partly because of the reduced capacity for endogenous neurogenesis observed in the aged brain (Seki & Arai 1991; Kempermann *et al.* 1998). To the extent that this impairment is due to age-related changes in the brain environment, as opposed to cell-autonomous defects in neural precursor cells, it might affect transplanted cells as well. For example, levels of several growth factors that stimulate neurogenesis decline with aging in neurogenic brain regions (Shetty *et al.* 2005). If transplanted NPCs are similarly dependent on these factors, neurotransplantation into the aged brain might prove futile.

We found previously that transplantation of hESC-derived, nestin^{+}/SOX1^{+}/calbindin^{-}/GFAP^{-}/OX4^{-} NPCs, together with Matrigel scaffolding, into young adult Sprague-Dawley rat brain 3 wks after MCAO reduced infarct volume and improved neurobehavioral performance (Jin *et al.* 2010). The major finding of the present study is that transplantation of the same cells and scaffolding confers benefit in both young adult (3-mo-old) and aged (24-mo-old) Fisher 344 rats. Infarct volume was larger and behavioral function more impaired in older rats. However, the magnitude of reduction in infarct volume afforded by NPC transplantation was comparable (~50% in 3- and ~40% in 24-mo-old animals). In addition, although older rats also showed more severe behavioral deficits, they also demonstrated more marked improvement following transplantation, perhaps because behavioral deficits were largely resolved by 6–8 wks post-transplant in younger animals.

A notable feature of the response to NPC transplantation is that it occurred even though transplantation was delayed until 3 wks after ischemia. The option for delayed treatment is clinically important because current therapy for stroke is only effective for a few hours after the event. Most previous studies of NPC transplantation in rodents have employed post-ischemic intervals of 1 wk or less, an exception being a study in which medial ganglionic eminence cells from E15 rat embryos were transplanted 2 wks post-MCAO (Daadi *et al.* 2009a). Some growth factors can also be administered up to several days after MCAO in rodents and produce improvement in infarct volume, behavior or both (Greenberg & Jin 2006). In one case, transforming growth factor-α given to rats 4 wks after MCAO was still capable of improving behavioral outcome (Guerra-Crespo *et al.* 2009). However, none of these studies involved aged animals.

In this and our prior study (Jin *et al.* 2010) of NPC transplantation after cerebral ischemia in rats, we did not treat the recipients with immunosuppressive drugs. Some (Ishibashi *et al.* 2004; Kelly *et al.* 2004; Daadi *et al.* 2009b) but not other (Chu *et al.* 2004; Chu *et al.* 2005; Daadi *et al.* 2010) previous studies have employed immunosuppression with cyclosporine A, although none has compared immunocompetent and immunosuppressed animals. Whether outcome would have been even better had our rats been immunosuppressed is impossible to say, but several factors argue against the need for immune suppression in this setting (Barker & Widner 2004). First, the brain is a relatively immunoprivileged site, due partly to the blood-brain barrier, a paucity of resident antigen-presenting cells, and sparse lymphatic drainage. Second, cell-suspension—as opposed to than solid tissue—grafts tend to evoke lesser immune responses. Third, intaparenchymal transplants (and perhaps especially transplants into poorly vascularized brain tissue) appear less immunogenic than, e.g., periventricular transplants. Fourth, transplantation into sites of pre-existing astro- and microglial activation may be associated with enhanced trophic support and improved graft survival (Duan *et al.* 1998). Finally, graft rejection in brain may occur over a longer time course than is typically monitored in rodents (Brevig *et al.* 2000). The role of aging in transplant rejection is complex, as observed in clinical studies. Immune function is impaired by aging, and elderly transplant recipients have a reduced incidence of acute rejection, although the risk of chronic rejection is increased and the adverse effects of immunosuppressive therapy are more common (Saxena *et al.* 2009). It is, therefore, possible that immunosenescence contributed to the success of NPC transplantation in our aged rats.

The mechanism through which NPC transplantation reduces infarct size and improves functional outcome after experimental cerebral ischemia is elusive. Cell replacement may be a contributing factor, but transplanted cells also release factors with neuroprotective or neurotrophic effects. Vascular endothelial growth factor (VEGF), for example, has been implicated in the protective effect of murine NPCs against MCAO in mouse brain, which is blocked by a VEGFR2 receptor antagonist and by a soluble VEGFR2 decoy receptor (Harms *et al.* 2010). In another study (Zhu *et al.* 2005), overexpression of VEGF in transplanted rat NPCs enhanced the effect of transplants on neurological function. Overexpression of other growth factors—including neurotrophin-3 (Zhang *et al.* 2008), fibroblast growth factor-2 (FGF-2) (Jenny *et al.* 2009), and glial cell line-derived neurotrophic factor (Chen *et al.* 2009)—in rat NPCs has also been reported to potentiate the effects of NPC transplantation after MCAO in rats.

EXPERIMENTAL PROCEDURES

Distal MCAO

Aged (24-mo-old) and young adult (3-mo-old) male Fisher 344 rats were obtained from the National Institute on Aging aged rodent colony. All animal procedures were conducted in accordance with National Institutes of Health (NIH) guidelines and with the approval of the Institutional Animal Care and Use Committee. Male rats were anesthetized with 4% isoflurane in 70% N_2O/30% O_2 using a mask. Permanent distal MCAO was performed as previously described (Nawashiro *et al.* 1997; Won *et al.* 2006). In brief, a 2-cm incision was made between the left orbit and tragus under the surgical microscope. The temporal muscle was retracted laterally and a 3-mm diameter craniotomy was made just rostral to the foramen ovale. The dura was incised with a 26–gauge needle and the distal MCA was exposed. The left MCA was occluded by electrocoagulation without damaging the brain surface. Interruption of blood flow was confirmed visually under the microscope, the temporal muscle was repositioned, and the skin was closed. Rectal temperature was measured and maintained at 37±0.2°C with a heating blanket. In sham-operated controls the MCA was visualized but not occluded.

Cell culture

NPCs derived from hESC line BG01 were obtained from Aruna Biomedical Inc. (Athens, GA). Cells were seeded on polyornithine- and laminin-coated plastic dishes and cultured in proliferation medium, consisting of Neurobasal medium with B27 supplementation containing 2 mM L-glutamine and 50 μg/ml Pen Strep (all from Invitrogen), and 10 ng/ml leukemia inhibitory factor and 20 ng/ml FGF-2 (both from R&D Systems) (Shin *et al.* 2006). Cells were propagated further in proliferation medium and, upon reaching 90–100% confluence, were triturated to detach them from dishes. After centrifugation at 200×*g* for 4 min, cells were resuspended in fresh medium and replated.

Cell transplantation

Cells were transplanted 3 wks after induction of focal ischemia. Rats (n=6–10 per group) were re-anesthetized with 4% isoflurane in 70% N_2O/30% O_2 and placed in stereotaxic frames with a rat head holder. Burr holes were drilled with a dental drill, which was irrigated continuously with saline at room temperature to prevent overheating of the underlying cortex. NPCs (1.2×10^5 cells/μl) were mixed with 15 μl of Matrigel suspension and immediately injected with a Hamilton syringe into the cortical infarct cavity over 5 min. The needle was then left in place for an additional 15 min before being slowly withdrawn. Control rats received injections of aCSF vehicle. Bone wounds were closed with bone wax, anesthesia was discontinued, and rats were returned to their cages. Eight weeks post-transplant, they were perfused with 0.9% saline and 4% paraformaldehyde in PBS (pH 7.5) to measure infarct volume.

Measurement of infarct volume

Brains were removed and 40-μm coronal sections were prepared and stained with cresyl violet. Infarct area was measured by a blinded observer using the NIH Image program, and areas were multiplied by the distance between sections to obtain the respective volumes. Infarct volume was calculated as a percentage of the volume of the contralateral hemisphere, as previously described (Swanson *et al.* 1990).

Cylinder test

The cylinder test (Schallert *et al.* 2000) was used to measure asymmetry of forelimb use 6 and 8 wks post-transplant (n=6–10 rats per group). Twenty movements were recorded by a blinded examiner during a 10-min testing session, and rated as involving the affected (right, R), unaffected (left, L) or both (B) limbs. Asymmetry of forelimb use was scored as (L−R)/(L+R+B), with higher scores reflecting preferential use of the unaffected limb and, therefore, more severe impairment.

Elevated body swing test (EBST)

Asymmetric motor behavior was also measured 6 and 8 wks post-transplant (n=6–10 rats per group) using the EBST (Borlongan & Sanberg 1995). Rats were held by the base of the tail and raised 10 cm above the testing surface. The initial direction of body swing (turning of the upper body by >10° to either side) was recorded by a blinded examiner in three sets of ten trials, performed over 5 min. The number of turns in each direction (right, R or left, L) was recorded, and the percentage of turns made away from the ischemic hemisphere (percent right-biased swing, normally 50%) was calculated as R/(R+L) × 100%.

BrdU administration

BrdU (50 mg/kg in saline) was given by the intraperitoneal route twice daily 24 hr before rats were euthanized (8 wks post-transplant). Brains were perfused with saline and 4% paraformaldehyde in PBS and embedded in paraffin.

Immunohistochemistry

Brain sections were embedded in paraffin and 6-μm coronal and sagittal sections were prepared. Immunohistochemistry and double immunostaining were performed as described previously (Jin *et al.* 2002; Jin *et al.* 2003). To detect BrdU-labeled cells, paraffin sections were treated with 50% formamide, 280 mM NaCl, and 30 mM sodium citrate at 65°C for 2 h, incubated in 2 M HCl at 37°C for 30 min, and rinsed in 0.1 M boric acid (pH 8.5) at room temperature for 10 min. Primary antibodies were affinity-purified goat polyclonal anti-doublecortin (DCX; Santa Cruz Biotechnology; 1:500), sheep polyclonal anti-BrdU (Biodesign; 1:1000), mouse monoclonal anti-human nuclei (HuN, Chemicon; 1;300), rabbit polyclonal anti-βIII tubulin (TUJ-1, Covance, Berkeley; 1:1000), rabbit anti-cleaved caspase-3 (Cell Signaling Technology; 1:200), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, Sigma-Aldrich; 1:400), and rabbit polyclonal anti-calbindin (Upstate; 1:400). Secondary antibodies were Alexa Fluro 488-, or 594- or 647-conjugated donkey anti- goat, anti- mouse or anti- rabbit IgG (Molecular Probes; 1:200–500). Fluorescence signals were detected using an LSM 510 NLO Confocal Scanning System mounted on an Axiovert 200 inverted microscope (Carl Zeiss Ltd.) equipped with a two-photon Chameleon laser (Coherent Inc.). Images were acquired using LSM 510 Imaging Software (Carl Zeiss Ltd). Controls included omitting or preabsorbing primary or omitting secondary antibody.

Statistical Analyses

Quantitative data were expressed as mean ± SEM from the indicated number of experiments. Behavioral data were analyzed by two-way analysis of variance (ANOVA) with repeated measures, followed by *post-hoc* multiple comparison tests (Fisher PLSD or Student's paired *t* test with the Bonferroni correction). Infarct volume data were analyzed by one-way ANOVA followed by Fisher PLSD *post-hoc* tests. p values <0.05 were considered significant.

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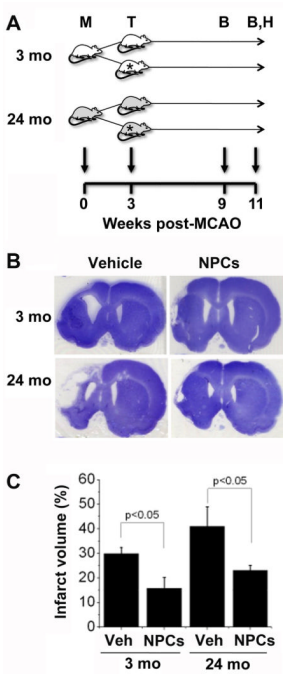

Figure 1. Infarction volume in young adult and aged rats after MCAO and NPC transplantation

(A) Experimental design: 3- and 24-mo-old rats underwent MCAO (M), followed 3 wks later by transplantation (T) of vehicle or NPCs; behavioral testing (B) was conducted 6 and 8 wks after transplantation, and rats were euthanized at 8 wks for histological studies (H). Stars indicate recipients of NPC transplants. **(B)** Cresyl violet-stained coronal rat brain sections 8 wks after transplantation of vehicle (Veh) or NPCs (Cells) into young adult (3-mo-old, 3M) and aged (24-mo-old, 24M) rats. **(C)** Infarct volume (% of contralateral hemisphere) was reduced after NPC transplantation in both age groups. Data are means ± SEM from 6–10 rats per group.

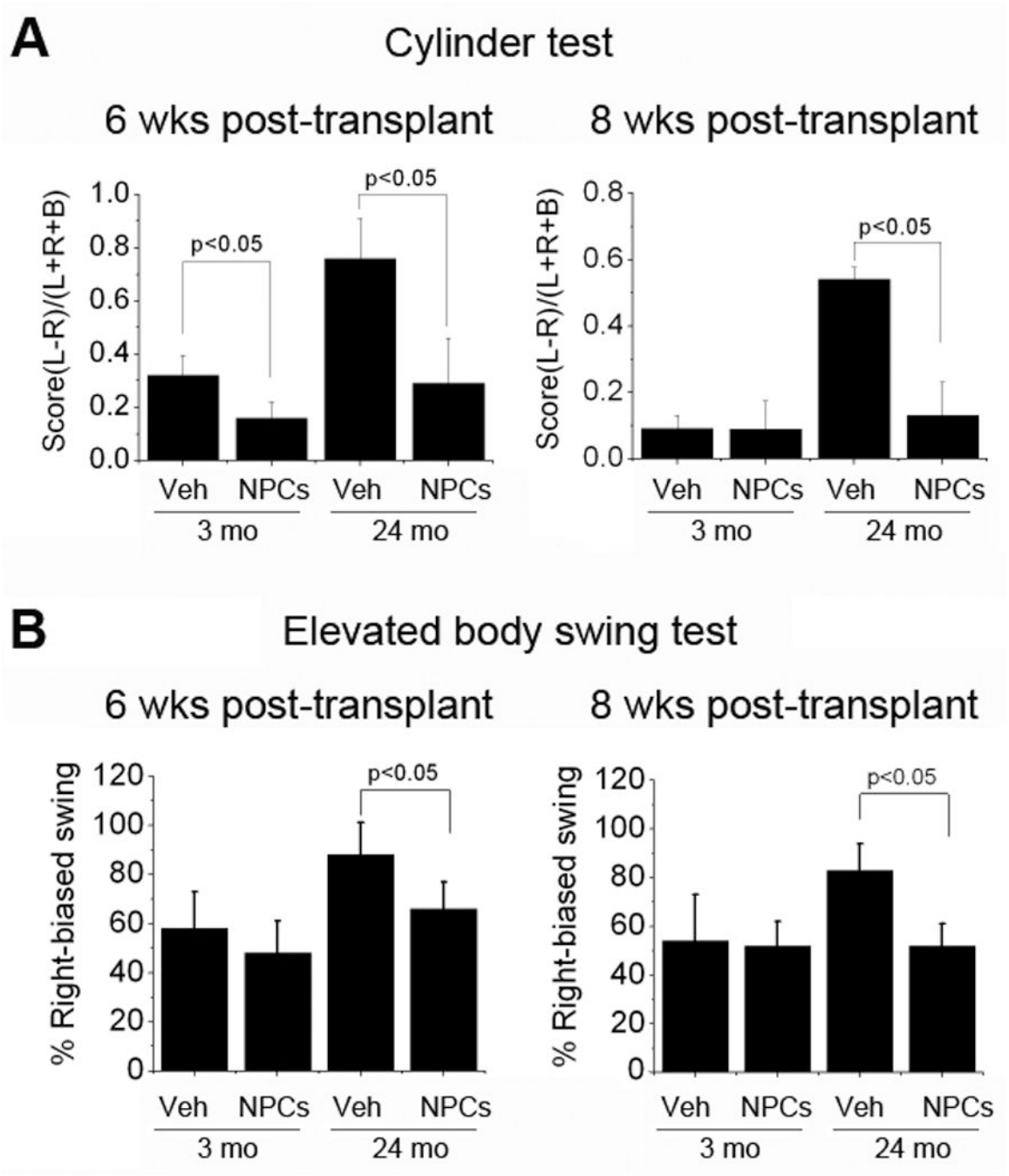

Figure 2. Behavioral testing in young-adult and aged rats after MCAO and NPC transplantation

Testing was conducted 6 and 8 wks after transplantation of vehicle (Veh) or NPCs (Cells) into young adult (3-mo-old, 3M) and aged (24-mo-old, 24M) rats. **(A)** Cylinder test: asymmetry of forelimb use was scored as (L−R)/(L+R+B), where L=left, R=right, and B=both, with higher scores reflecting preferential use of the unaffected limb and, therefore, more severe impairment. **(B)** Elevated body swing test: The number of turns in each direction (L=left, R=right) was recorded, and the percentage of turns made away from the ischemic hemisphere (percent right-biased swing, normally 50%) was calculated as R/(R+L) × 100%. Data are means ± SEM from 6–10 rats per group.

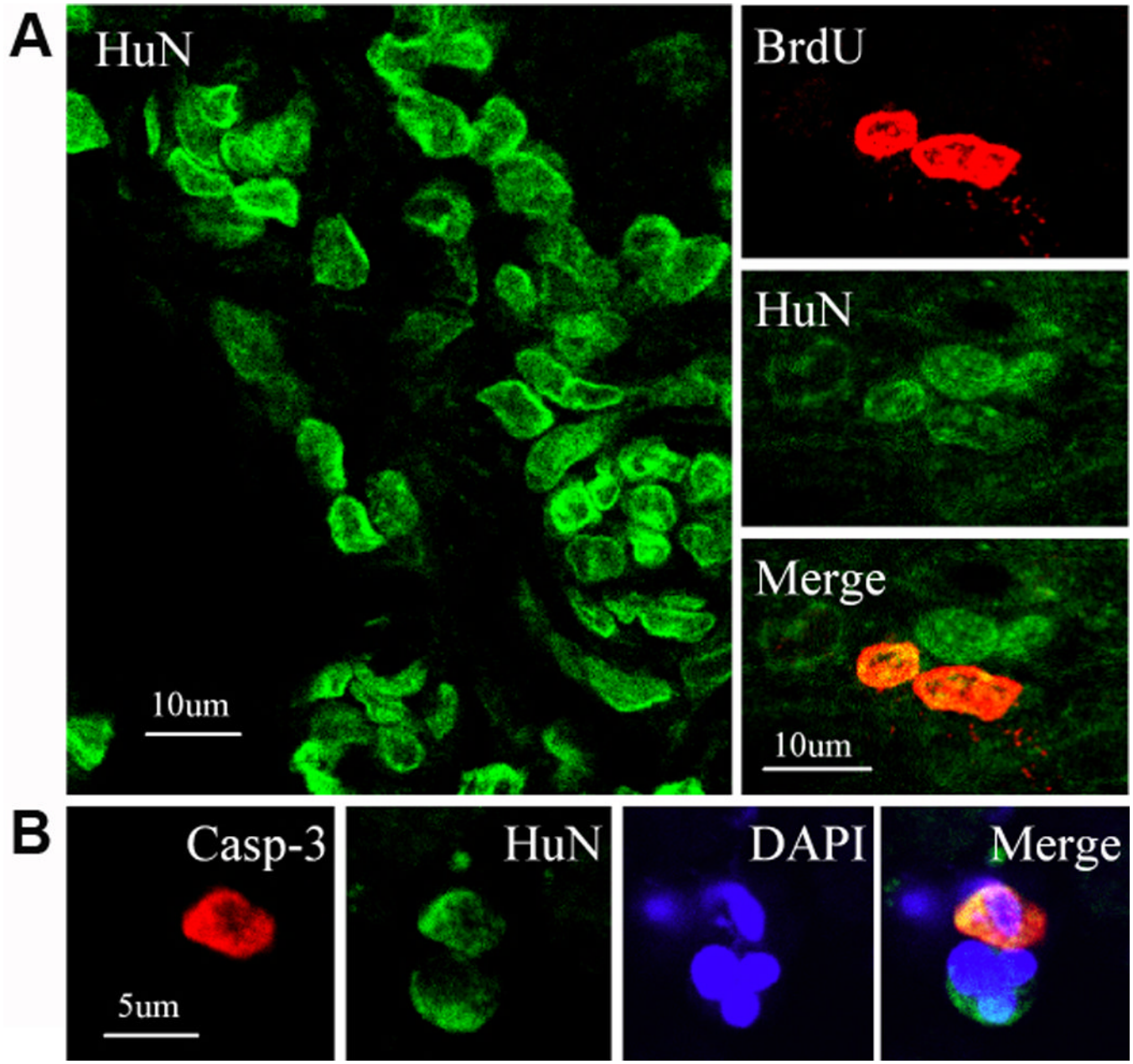

Figure 3. Survival of transplanted NPCs in post-ischemic aged rat brain

Sections were obtained 8 wks after transplantation of NPCs into aged (24-mo-old) rats. **(A)** Human nuclei (HuN)-positive (green) cells were present at the transplant site (left), and in some cases were labeled with BrdU (red), suggesting recent cell division (right). **(B)** Some HuN-positive (green) cells also expressed activated caspase-3 (red), consistent with ongoing caspase-dependent cell death. DAPI (blue) was used to counterstain nuclei.

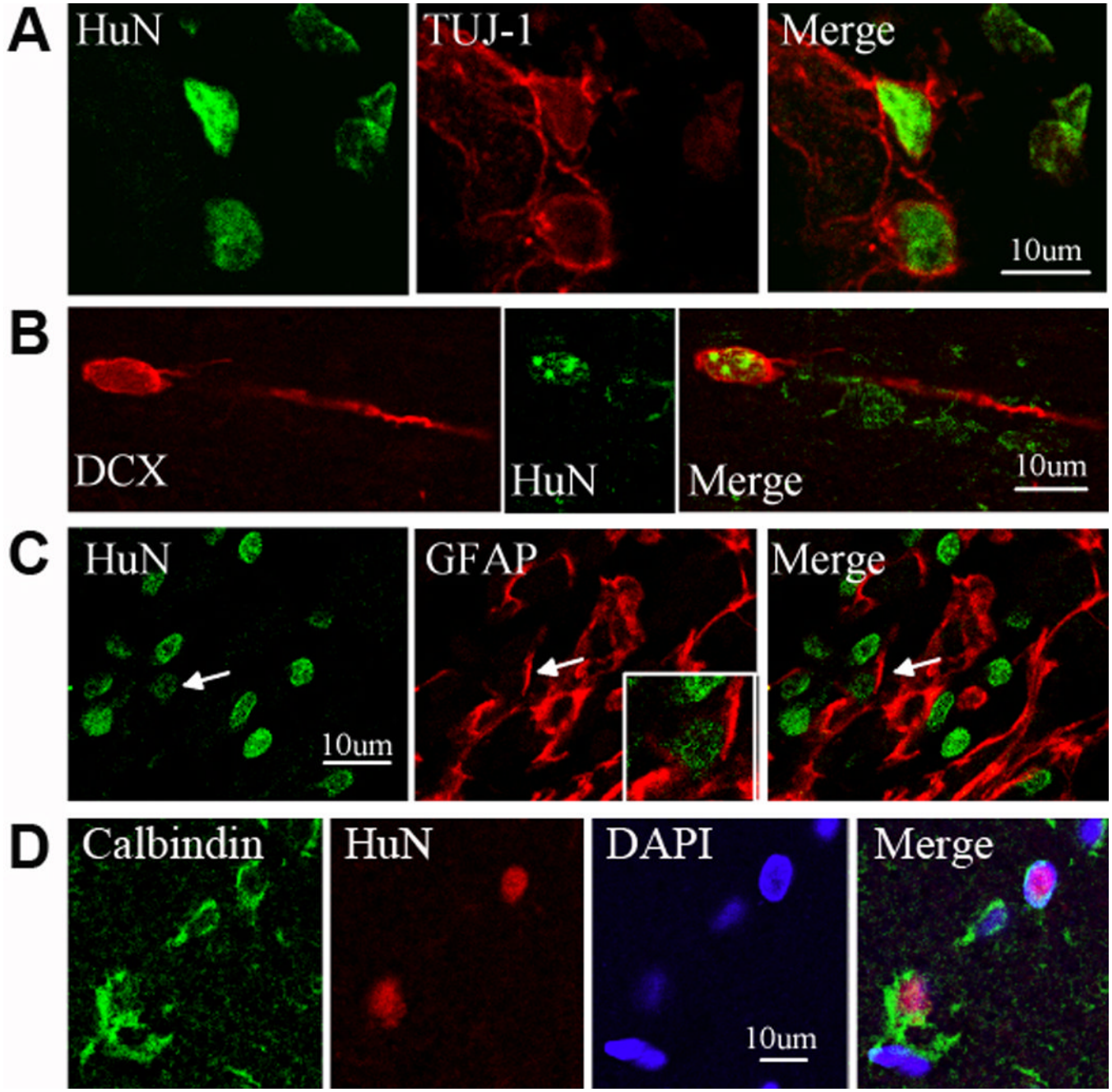

Figure 4. Phenotypis fate of transplanted NPCs in post-ischemic aged rat brain

Sections were obtained 8 wks after transplantation of NPCs into aged (24-mo-old) rats. Transplanted (HuN-positive, green) cells co-expressed the neuronal lineage markers βIII tubulin (TUJ-1 antibody) **(A)** or DCX **(B)**, the astroglial marker GFAP **(C)**, or the mature neuronal marker calbindin **(D)**. DAPI (blue) was used to counterstain nuclei.