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Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain

Robert H. Andres,[1] Nobutaka Horie,[1] William Slikker,[1] Hadar Keren-Gill,[1] Ke Zhan,[2] Guohua Sun,[1] Nathan C. Manley,[1] Marta P. Pereira,[1] Lamiya A. Sheikh,[3] Erin L. McMillan,[4] Bruce T. Schaar,[1] Clive N. Svendsen,[5] Tonya M. Bliss[1,*] and Gary K. Steinberg[1,*]

1 Department of Neurosurgery, Stanford Stroke Centre and Stanford Institute for Neuro-Innovation and Translational Neurosciences, Stanford University School of Medicine, Stanford, CA, USA
2 Molecular Imaging Program at Stanford, Department of Radiology, Stanford University, Stanford, CA, USA
3 Department of Health Research and Policy, Stanford University, Stanford, CA, USA
4 University of Wisconsin-Madison, Madison, WI, USA
5 Regenerative Medicine Institute, Cedars-Sinai Medical Centre, Los Angeles, CA, USA

*These authors contributed equally to this work.

Correspondence to: Tonya M. Bliss, PhD,
Department of Neurosurgery,
1201 Welch Road,
Stanford,
CA 94305-5487, USA
E-mail: tbliss1@stanford.edu

Correspondence may also be addressed to: Gary K. Steinberg, MD PhD. Department of Neurosurgery, 300 Pasteur Drive, R281 Stanford, CA 94305-5327, USA. E-mail: gsteinberg@stanford.edu

Stem cell transplantation promises new hope for the treatment of stroke although significant questions remain about how the grafted cells elicit their effects. One hypothesis is that transplanted stem cells enhance endogenous repair mechanisms activated after cerebral ischaemia. Recognizing that bilateral reorganization of surviving circuits is associated with recovery after stroke, we investigated the ability of transplanted human neural progenitor cells to enhance this structural plasticity. Our results show the first evidence that human neural progenitor cell treatment can significantly increase dendritic plasticity in both the ipsi- and contralesional cortex and this coincides with stem cell-induced functional recovery. Moreover, stem cell-grafted rats demonstrated increased corticocortical, corticostriatal, corticothalamic and corticospinal axonal rewiring from the contralesional side; with the transcallosal and corticospinal axonal sprouting correlating with functional recovery. Furthermore, we demonstrate that axonal transport, which is critical for both proper axonal function and axonal sprouting, is inhibited by stroke and that this is rescued by the stem cell treatment, thus identifying another novel potential mechanism of action of transplanted cells. Finally, we established *in vitro* co-culture assays in which these stem cells mimicked the effects observed *in vivo*. Through immunodepletion studies, we identified vascular endothelial growth factor, thrombospondins 1 and 2, and slit as mediators partially responsible for stem cell-induced effects on dendritic sprouting, axonal plasticity and axonal transport *in vitro*. Thus, we postulate that human neural progenitor cells aid recovery after stroke through secretion of factors that enhance brain repair and plasticity.

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Keywords: stroke; transplantation; brain rewiring; APP; dendrites

Abbreviations: BDA = biotinylated dextran amine; GAP-43 = growth-associated protein-43; NPC = neural progenitor cell; SPARC = secreted protein acidic and rich in cysteine; VEGF = vascular endothelial growth factor

Introduction

Stem cell therapy has emerged as a promising new experimental treatment strategy for stroke and other diseases of the CNS. Transplanted neural- or mesenchymal-derived progenitor cells are known to survive and migrate in the rodent brain after ischaemic damage and to improve functional recovery (Chen *et al.*, 2001, 2003; Kelly *et al.*, 2004; Guzman *et al.*, 2008; Hicks *et al.*, 2009; Darsalia *et al.*, 2010). Initial clinical trials demonstrated the feasibility of cell therapy in patients with stable and persistent stroke deficits (Bang *et al.*, 2005; Kondziolka *et al.*, 2005; Savitz *et al.*, 2005), and currently six new clinical trials are in progress (Bliss *et al.*, 2010). However, despite many preclinical studies showing that cell transplantation can improve recovery from stroke, the mechanisms mediating recovery are unknown.

One hypothesis is that stem cells, through secretion of factors, act to enhance endogenous repair mechanisms normally activated in the brain after stroke such as vascular repair, immunomodulation, endogenous neurogenesis and brain plasticity (Arvidsson *et al.*, 2002; Taguchi *et al.*, 2004; Liu *et al.*, 2008; Ohtaki *et al.*, 2008; Bliss *et al.*, 2010; Li *et al.*, 2010; Horie *et al.*, 2011). Brain plasticity is thought to play a major role in the spontaneous recovery observed in patients and animals after stroke with the dramatic reorganization and rewiring of surviving circuits enabling the healthy brain to take over the function of the damaged area (Dancause, 2006; Murphy and Corbett, 2009; Benowitz and Carmichael, 2010). Functional imaging and stimulation studies in patients have shown a remapping of the brain after stroke which, at least in the first few weeks, indicates recruitment of both ipsi- and contralesional brain areas. Animal studies yielded similar results (Dijkhuizen *et al.*, 2003; Takatsuru *et al.*, 2009) and suggest this gross remapping is caused by local and long distant changes in axonal sprouting and dendritic arborization (Jones and Schallert, 1992; Gonzalez *et al.*, 2003), which we define here as structural plasticity.

Recovery from neurological deficits will also depend on restoration of axonal function. Many circuits remain intact after stroke, but are potentially impaired due to disruption of axonal transport (Kataoka *et al.*, 1989; Valeriani *et al.*, 2000; Wakita *et al.*, 2002); this is particularly relevant to white matter tract injury. Axonal transport is critical for neuron function; transport of molecules and organelles between the cell body and the axon terminal is essential for neurotransmission. Furthermore, axonal transport is critical for the relay of molecules that signal axonal sprouting or degeneration, and synapse formation or retraction (Coleman, 2005; Salehi *et al.*, 2009). Therefore, restoration of impaired axonal transport after stroke is likely to be key for both the proper functioning of existing axons and for plasticity changes such as axonal sprouting and synaptogenesis.

The factors involved in signalling axonal and dendritic changes after stroke are not really understood but many molecules are known to promote neurite sprouting either in culture or *in vivo* (Carmichael *et al.*, 2005). Such molecules include neurotrophic factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (Himmelseher *et al.*, 1997; Rosenstein *et al.*, 2003), extracellular matrix molecules like the thrombospondins and secreted protein acidic and rich in cysteine (SPARC) (Osterhout *et al.*, 1992; Au *et al.*, 2007; Liauw *et al.*, 2008) and factors important for neurite growth and guidance during development like Slit (Lin and Isacson, 2006). Neural progenitor cells express many such factors known to influence neurite plasticity (Wright *et al.*, 2003) and thus have the potential to enhance structural plasticity after stroke. Therefore, in the present study, we determined the effects of transplanted human neural progenitor cells (NPCs) on structural plasticity and axonal transport in the ischaemic rat brain and correlated this with human NPC-induced recovery. Furthermore, we investigated the potential of several human NPC-secreted factors to induce such plasticity and transport changes *in vitro*.

Materials and methods

Human neural progenitor cell culture

Human NPCs, initially isolated from foetal cortical brain tissue at 13.5 weeks gestation (M031 clone), were cultured as neurospheres in Stemline™ Neural Stem Cell Expansion Medium (Sigma) supplemented with 20 ng/ml epidermal growth factor (Sigma) and 10 ng/ml leukaemia inhibitory factor (Chemicon) as previously described (Svendsen *et al.*, 1998). For transplantation and *in vitro* experiments, human NPCs (passages 16–25) were dissociated to a single cell suspension by incubation at 37°C with Accutase (10 min; Sigma), trypsin inhibitor (5 min; Sigma) and DNase (10 min; Sigma), followed by gentle trituration.

Stroke surgery and cell transplantation

Animal procedures were approved by Stanford University's Administrative Panel on Laboratory Animal Care. T cell-deficient adult male Nude rats (Cr:NIH-RNU 230 ± 30 g; NCI-Frederick Cancer Research) were subjected to permanent distal middle cerebral artery occlusion with 0.5 h bilateral common carotid artery occlusion as described (Kelly *et al.*, 2004) under isoflurane anaesthesia. Ampicillin (1 mg/ml) was administered orally 3 days before stroke surgery to 7 days post-transplantation surgery. At 7 days post-distal middle cerebral artery occlusion, three 1.0 µl deposits of human NPCs (1×10^5 cells/µl), or vehicle (0.9% saline), were injected into the ipsilesional cortex as described (Kelly *et al.*, 2004): (i) anterior–posterior (A–P), + 1.0; medial–lateral (M–L), −1.2; dorsal–ventral (D–V), −2.7; (ii) A–P, −0.26; M–L, −1.2; D–V, −2.6; and (iii) A–P, −1.8; M–L, −1.0; D–V, −1.8. Rats were housed in groups of two or three in cages containing a plastic toy (polyvinyl chloride tubing), with free access to food and water. A total of three rats died in the first 24 h after induction of stroke. Otherwise, no animals were excluded from the study.

Behavioural testing

Behaviour was tested preoperatively for baseline performance and repeated weekly for 6 weeks post-stroke by an investigator blinded to treatment ($n = 12$ per group, except cylinder test $n = 6$). Animals were randomized at 1 week post-stroke, prior to treatment, based on their behaviour scores in the vibrissae-elicited forelimb placing test. No animals were excluded from the study. (i) Vibrissae-elicited forelimb placing test: 10 trials of the vibrissae-evoked forelimb placing test were carried out on each side as described previously (Schallert *et al.*, 2000); (ii) cylinder test (Schallert *et al.*, 2000): rats were placed in a plastic cylinder and the number of times they reared and touched the cylinder in a weight-bearing fashion with the left, right, or both forelimbs was counted for 20 hits. The right:left ratio was calculated; (iii) elevated body swing test (Borlongan and Sanberg, 1995): rats were lifted by the proximal tail 3 cm above the table, the direction of swing in order to reach upward was recorded for 10 trials; (iv) postural reflex test (Bederson *et al.*, 1986): animals were suspended by the proximal tail, 1 m above the ground and slowly lowered. Symmetric touch-down with both forelimbs scored 0 points and testing ended. Animals with abnormal postures (rotating body, flexing of limbs) were placed on the table and gently pushed side-to-side. Rats resistant to pushing scored 1 point, animals unable to resist scored 2 points.

Tissue processing and immunostaining

Rats were perfused as previously described (Kelly *et al.*, 2004), brains and cervical spinal cords removed and 30 μm or 20 μm coronal sections cut, respectively. Immunostaining was performed conventionally (Kelly *et al.*, 2004) using primary and secondary antibodies as described in the Supplementary material, followed by confocal microscopy analysis. To assess the human NPC differentiation profile, *Z*-stacks were acquired and 100 or more human nuclear antigen-positive cells in the peri-infarct area were scored for each marker (Nestin, neuronal class III β-Tubulin, glial fibrillary acidic protein, NG2, Olig2). Pixel intensity of growth-associated protein-43 (GAP-43) staining was analysed using ImageJ in six brain slices encompassing the entire lesion. All histological analyses were carried out in a blinded fashion.

Analysis of axonal transport *in vivo*

Immunohistochemistry for axonal amyloid precursor protein was performed as previously described (Stone *et al.*, 2000; Supplementary material) using rabbit anti-amyloid precursor protein (C-terminus; 1:2000; Invitrogen) with the mouse anti-neural filament (anti-SMI312) antibody (1:1000; Sternberger Monoclonals). Brains were analysed (blinded) at 3 weeks ($n = 6$) and 12 weeks ($n = 12$) after transplantation. Confocal images were acquired from six sections per animal (~500 μm apart) in two regions of interest per section, namely the ipsi- and contralesional genu of the corpus callosum. The total number of SMI312-positive fibres per region of interest and the ratio of amyloid precursor protein/SMI312-co-localizing axons were analysed using the 'Puncta Analyzer' plugin for NIH ImageJ software, as described previously (Liauw *et al.*, 2008; Supplementary material), and averaged over the six sections.

Analysis of infarct size and corpus callosum thickness

Infarct size was determined by an investigator blinded to treatment, on six cresyl violet stained sections taken at 540 μm intervals between the levels +1.2 to −1.6 mm from bregma and expressed as percentage of the contralateral, non-ischaemic hemisphere as described previously (Kelly *et al.*, 2004). Corpus callosum thickness was measured (blinded) at the midline on bregma level slices ($n = 12$ per group, except naïve animals, $n = 5$).

Dendritic analysis

Rats were anaesthetized with isoflurane ($n = 4$–5 per group at 2 weeks post-transplantation, $n = 5$ per group at 4 weeks post-transplantation), the brains were removed and stained using a Rapid GolgiStain™ Kit (FD NeuroTechnologies) and 150 μm coronal sections cut. Layer V pyramidal neurons were analysed (blinded) in the region between the lesion and the human NPC graft and the equivalent location in the contralesional cortex, i.e. between bregma and bregma −1.2 mm, between the dorsal peak of the corpus callosum and up to 4 mm from the midline. Five neurons per hemisphere per animal were analysed. To be included, neurons had to be well impregnated, in full view with no overlapping blood vessels or astrocytes, appear intact and in the plane of section. The length of each dendritic branch was determined using the measuring tool on the StereoInvestigator software (MicroBrightField) and following the dendrite through the *Z*-axis; the number of branches was tabulated at the same time. Branch order was analysed as shown in Fig. 2 (Coleman and Riesen, 1968).

Axonal tracing studies

At 2 ($n = 6$) or 4 weeks ($n = 12$) post-transplantation, rats were injected with 0.2 μl of the anterograde axonal tracer biotinylated dextran amine (BDA, molecular weight 10 000, 0.1 μg/μl; Molecular Probes) at 0.1 μl/min into the contralesional layer V cortex at: (i) A–P, −1.0; M–L, −1.3; D–V, −1.8; (ii) A–P, −1.0; M–L, −1.8; D–V, −1.8; (iii) A–P, −0.5; M–L, −1.3; D–V, −1.8; and (iv) A–P, −0.5; M–L, −1.8; D–V, −1.8. The needle was left *in situ* for 5 min then slowly removed. After 1 week brains and cervical spinal cord were processed for immunohistochemistry: after 30 min permeabilization with digitonin (100 μg/ml; Calbiochem), BDA was detected using streptavidin-conjugated antibody (Alexa Fluor 546, 1:200; Molecular Probes). Biotinylated dextran amine-positive fibres were determined by a stereological approach (Supplementary material). In brief, confocal images were acquired from defined regions of interest in six defined brain slices encompassing the entire ischaemic area, and from the dorsal funiculus of the spinal cord at level C5. BDA-positive fibre densities were calculated using ImageJ software by determining the number of pixels above a set intensity threshold. The values were normalized to the number of BDA-positive cell bodies. Image acquisition and analysis were done in a blinded fashion. Quantification of BDA-positive fibres was validated by a second, manual counting process as previously described (Kartje *et al.*, 1999; Chen *et al.*, 2002) (Supplementary material).

RNA extraction and quantitative polymerase chain reaction analysis

Brains were removed from human NPC- or vehicle-transplanted ischaemic rats 1 week post-transplantation ($n = 4$ per group). RNA was extracted from the transplantation sites and human gene expression determined using human-specific Taqman Gene Expression Assays (Applied Biosystems). Refer to the Supplementary material for details.

In vitro dendritic and axonal outgrowth assays

Primary cortical cultures from Sprague-Dawley rat E14 embryos (Charles River Laboratories) were prepared as described (Andres *et al.*, 2005; Supplementary material). After 3 days *in vitro* human NPCs (180 000 cells) or vehicle were added to the primary cultures in cell culture inserts (Falcon 353095). For immunodepletion studies, neutralizing antibodies were added to the medium in the wells and inserts at 5 μg/ml from Days 3–7 *in vitro* (see Supplementary material). At Day 7 *in vitro*, the cortical cultures were fixed and axons labelled with anti-SMI312 (1:1000) and dendrites with anti-microtubule-associated protein 2 (MAP2, 1:400; BD Pharmingen), followed by secondary antibodies (Alexa Fluor 488 or 546 conjugated goat anti-mouse, 1:250; Molecular Probes). Fluorescent images of randomly selected fields were acquired and neurite outgrowth and MAP2-positive cell numbers analysed using automatic neurite tracing software (HCA Vision V1.6.5, CSIRO). Minor processes measuring <6 μm were excluded from the axonal analysis. For neurite outgrowth assays, 42–458 individual cells from 3–6 independent experiments were analysed per group. For cell number analysis, 6–12 cultures were analysed per group from 3–6 independent experiments.

Microfluidics axonal transport assay

E14 rat embryonic primary cortical cultures were grown in the 'cell body' chamber of a microfluidics device (Taylor *et al.*, 2005; Supplementary material). At Day 3 *in vitro*, 120 000 human NPCs or vehicle were added to the distal chamber. At Day 5 *in vitro*, when axons of the primary cultures had extended through the microgrooves, Alexa Fluor 488-conjugated dextran (0.1 mg/ml; molecular weight 10 000, anionic, Nr. D-22910, Invitrogen) was added to the cell body chamber, and anterograde transport of dextran-labelled vesicles was analysed as reported (Kunzi *et al.*, 2002). In brief, 2 h after dextran labelling, time-lapse fluorescent images were acquired every 12.5 s. Transport velocities of individual anterogradely transported vesicles were calculated if they were identifiable for at least six frames in a series. Vesicles with velocities <0.1 μm/s were discarded to exclude movements caused by Brownian motion (Kunzi *et al.*, 2002). Immunodepletion studies were carried out as described above with antibodies added to both the cell body and distal compartments. A total of 140–840 vesicles were analysed per group, from four independent experiments.

Statistical evaluation

Statistical analysis utilized GraphPad Instat (GraphPad Software) and JMP 8.0.1 (SAS Institute). *In vivo* dendritic data analysis consisted of repeated measures ANOVA using PROC GLM, a procedure in SAS, to compare treatment effects after adjusting for subject effects. ANOVA assumptions were examined with residual plots. For all other data, experimental groups were compared by ANOVA, followed by non-parametric *post hoc* Mann–Whitney test, or Student's *t*-test. Biotinylated dextran amine or amyloid precursor protein values (from the genu of the contralateral corpus callosum) were tested for correlation with functional recovery (defined as change in behaviour from Week 1 to Week 6 post-stroke) using the Spearman test; both the Spearman coefficient and significance values are given. Differences were considered statistically significant at $P < 0.05$. Values are presented as mean ± SEM.

Results

Transplanted human neural precursor cells survive in the ischaemic host brain, reduce white matter atrophy and enhance functional recovery

Human NPCs transplanted into the rat cortex 7 days post-stroke exhibited robust survival and migration towards the lesion at 5 weeks post-transplantation (Fig. 1A). The majority of the human NPCs stained with nestin (78.2 ± 6.0%) implying that most cells remained in an immature state at this time, consistent with previous work (Kelly *et al.*, 2004; Hicks *et al.*, 2009). A small proportion of cells expressed the astrocyte marker glial fibrillary acidic protein (17.2 ± 7.9) or the immature neuronal marker β-tubulin (neuronal class III β-Tubulin; 4.6 ± 2.2%) (Fig. 1B and C). No co-localization was observed with the oligodendroglial markers NG2 and Olig2. Lesion size was not significantly different between human NPC- and vehicle-treated rats at 5 weeks post-transplant (21.2 ± 3.7% and 15.3 ± 1.9%, respectively; Supplementary Fig. 1A), but atrophy of the corpus callosum was significantly reduced in human NPC-treated animals at this time point [corpus callosum thickness (μm): 222 ± 13.8 (vehicle) versus 277 ± 11.3 (human NPC); $P < 0.05$; Supplementary Fig. 1B]. Human NPC-grafted rats showed significantly enhanced functional recovery in three out of four behaviour tests compared with vehicle controls (MANOVA $P < 0.05$) which started between 3 and 4 weeks post-transplantation.

Human neural precursor cell transplantation promotes dendritic plasticity

Golgi-stained dendrites from layer V cortical pyramidal neurons (Fig. 2C) were analysed between the lesion and the graft (and the corresponding region of interest in the contralateral cortex). Human NPC treatment enhanced dendritic branching and total dendritic length at 2 weeks post-transplantation in both hemispheres compared with vehicle controls (Fig. 2A and B). Of note, vehicle injection appeared detrimental to dendritic plasticity in the injected (ipsilesional) hemisphere compared with non-treated stroke animals at this time point (Fig. 2A and B). Human NPC-induced dendritic changes in the contralesional hemisphere abated by 4 weeks post-transplant. In contrast, the effects in the ipsilesional hemisphere were sustained suggesting that proximity to the human NPCs is important to maintain enhanced dendritic plasticity. Changes in dendritic branching were more significant in basilar branches when compared with apical branches (Fig. 2D–F), and the greatest plasticity was observed in the middle order branches (branch orders 3–7) rather than in branches emanating from the soma or at the dendrite extremities (Fig. 2E and F).

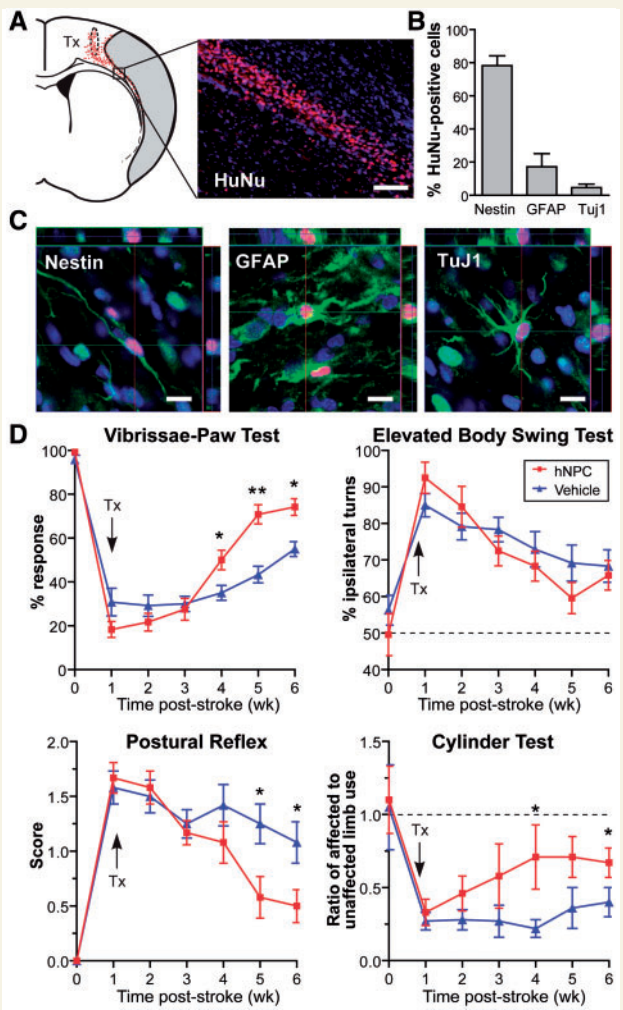

Figure 1 Differentiation fate of transplanted human NPCs and effect on behavioural recovery after stroke. (**A**) Human NPCs (human nuclear antigen$^+$ cells; red) exhibit good survival and migration towards the lesion at 5 weeks post-transplantation. Representative photomicrograph of human NPCs in the peri-infarct region. Grey-shaded area indicates the lesion. (**B**) Differentiation profile of the human NPCs at 5 weeks post-transplant. (**C**) Confocal images of differentiation markers co-localizing with human nuclear antigen in the peri-infarct area. (**D**) Human NPC-grafted animals have significantly improved functional recovery compared with vehicle-injected controls in three out of four behaviour tests; $*P < 0.05$, $**P < 0.01$. $n = 12$ per group, except cylinder test $n = 6$. Scale bars: **A** = 50 μm, **C** = 10 μm. GFAP = glial fibrillary acidic protein; HuNu = human nuclear antigen; TuJ1 = neuronal class III β-Tubulin; Tx = transplantation.

Human neural precursor cell transplantation promotes axonal rewiring after stroke

The anterograde axonal tracer biotinylated dextran amine (BDA) injected into the contralesional cortex was used to visualize axons. Human NPC-grafted rats, compared with vehicle-treated rats, appeared to increase axonal sprouting from the contralesional cortex to the ipsilesional hemisphere (Fig. 3A). This was first evident at 3 weeks post-transplantation with increased BDA-labelled fibre density in the corpus callosum and ipsilesional striatum of human NPC-treated animals (Fig. 3B). This effect was even more pronounced at 5 weeks post-transplantation with increased corticocortico, corticostriatal and corticothalamic sprouting as evidenced by significantly increased BDA-labelled fibre density in the relevant regions of interest as indicated in Fig. 3B and C. Unlike human NPC-treated animals, the vehicle-treated group showed no increase in BDA-positive fibres over time. Human NPC-grafted rats also showed enhanced corticospinal tract projections at 5 weeks post-transplantation with significantly increased BDA-labelled fibre density in the contralesional internal capsule and both the contra- and ipsilesional dorsal funiculus of the cervical spinal cord (Fig. 3D). Similar results were found when BDA was analysed by a second counting method (Supplementary material and Supplementary Fig. 2). To further substantiate human NPC-induced plasticity, we found that human NPC treatment significantly enhanced expression of the axonal growth cone protein GAP-43 in the corpus callosum and cortex of both hemispheres, with the largest increase in the ipsilesional cortex (Supplementary Fig. 1C). BDA labelling in the corpus callosum at 5 weeks post-transplantation positively correlated with functional recovery in the whisker-paw (Spearman correlation coefficient $\rho = 0.802$; $P = 0.001$) and cylinder tests (Spearman correlation coefficient $\rho = 0.642$; $P = 0.028$) when combining data from both vehicle and human NPC groups. Recovery in the whisker-paw test also positively correlated with the BDA signal in the injured corticospinal tract (Spearman correlation coefficient $\rho = 0.631$; $P = 0.032$). There was no correlation between lesion size and BDA signal. These data suggest that human NPC-induced axonal changes are important for human NPC-enhanced recovery.

Identification of human neural precursor cell-secreted factors that modulate dendritic and axonal plasticity

In a non-contact co-culture assay (Supplementary Fig. 3), human NPCs significantly increased (per neuron) dendritic branching (Fig. 4A and B), total dendritic length (Fig. 4A and C) and axonal length (Fig. 4A and D) of co-cultured cortical neurons, thus mimicking *in vitro* the human NPC-mediated effects on dendritic and axonal plasticity observed *in vivo*. To identify potential human NPC-secreted plasticity mediators, immunodepletion studies were done for specific candidate molecules known to be involved in neuronal plasticity and shown to be expressed in our human NPCs by microarray analysis (Wright *et al.*, 2003). Neutralization of thrombospondins 1 and 2 or human VEGF significantly reduced human NPC-induced dendritic branching and length, while neutralization of Slit only affected total dendritic length with no effect on dendritic branching (Fig. 4B and C). Human NPC-mediated axonal outgrowth was significantly reduced by neutralization of all the aforementioned factors (Fig. 4D); neutralization of

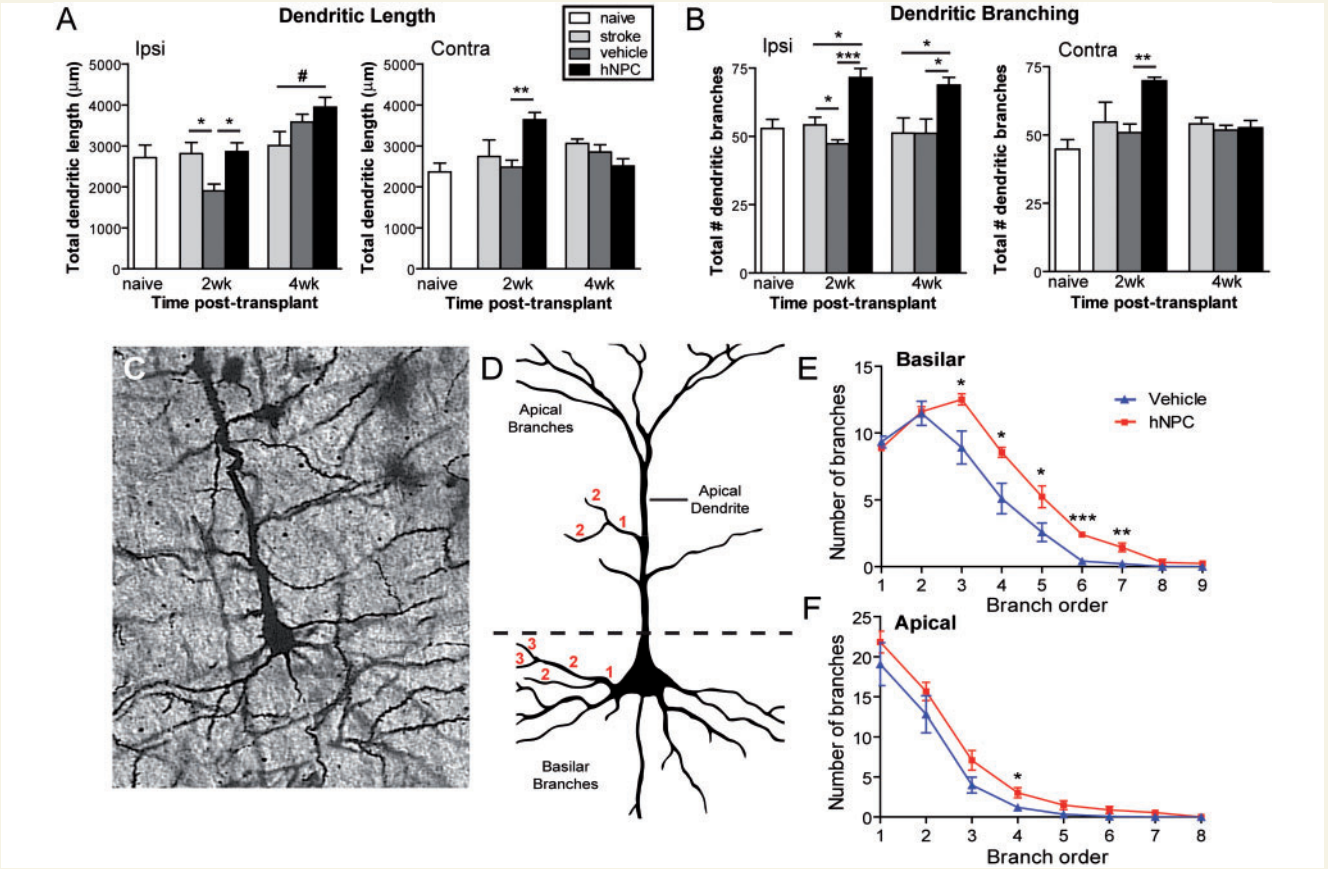

Figure 2 Human NPC transplantation enhances dendritic plasticity after stroke. (**A** and **B**) Human NPCs enhance dendritic length (**A**) and branching (**B**) at 2 weeks post-transplantation in both the ipsi- and contralesional cortex compared with vehicle-treated animals ($n = 4$–5 per group). These effects are sustained to 4 weeks post-stroke ($n = 5$ per group) only in the ipsilesional hemisphere. (**C**) Representative image of a golgi-stained layer V neuron. (**D**) Schematic illustrating how branch order is counted for apical and basilar dendrites. (**E** and **F**) At 4 weeks post-transplantation human NPCs enhance branching in the middle order branches in ipsilesional layer V neurons. This is more significant in basilar branches (**E**) compared with apical branches (**F**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#}P = 0.05$.

thrombospondins 1 and 2 also inhibited axonal growth in vehicle-treated cortical neurons. Depletion of SPARC had no effect on either dendritic or axonal morphology (Fig. 4B–D). The effects of the neutralizing antibodies were specific as isotype control antibodies had no effect ($P > 0.05$). Quantitative polymerase chain reaction analysis of the stroke brains 1 week post-transplantation revealed that at least four of these factors (VEGF, thrombospondins 1, 2 and SPARC) were expressed by the transplanted human NPCs (Table 1 and Supplementary Fig. 4), indicative of a potential role *in vivo*.

In addition to effects on neurite outgrowth *in vitro*, human NPCs enhanced survival of the primary neurons at Day 7 [245 ± 24 cells/mm^2 (−human NPCs) versus 348 ± 27 cells/mm^2 (+human NPCs); $P = 0.02$]. Depletion of thrombospondin 1 or human VEGF significantly reduced human NPC-induced neuron survival [218 ± 23 cells/mm^2 (thrombospondin 1); 260 ± 21 cells/mm^2 (human VEGF); $P < 0.05$ compared with human NPCs with no neutralization]. In contrast, depletion of thrombospondin 2, SPARC or Slit had no effect.

Transplanted human neural precursor cells reduce impairment of axonal transport post-ischaemia

Disruption of anterograde axonal transport was quantitatively assessed by measuring amyloid precursor protein accumulation in axons (labelled with the neurofilament marker SMI 312). There was significant accumulation of amyloid precursor protein in axons in the corpus callosum after stroke (Fig. 5A and B), consistent with previous reports of stroke impaired axonal transport (Valeriani *et al.*, 2000; Wakita *et al.*, 2002). Human NPC-grafted animals had significantly fewer amyloid precursor protein-positive axons at 3 weeks compared with vehicle controls (Fig. 5B), and this difference was further enhanced by 5 weeks suggesting that human NPCs enhance recovery of axonal transport. Furthermore, at 5 weeks human NPC animals had more axons in the corpus callosum as determined by the number of SMI312 puncta (Fig. 5C). Together, these data imply that human NPC-treated animals have more functioning axons (i.e. axons without impaired

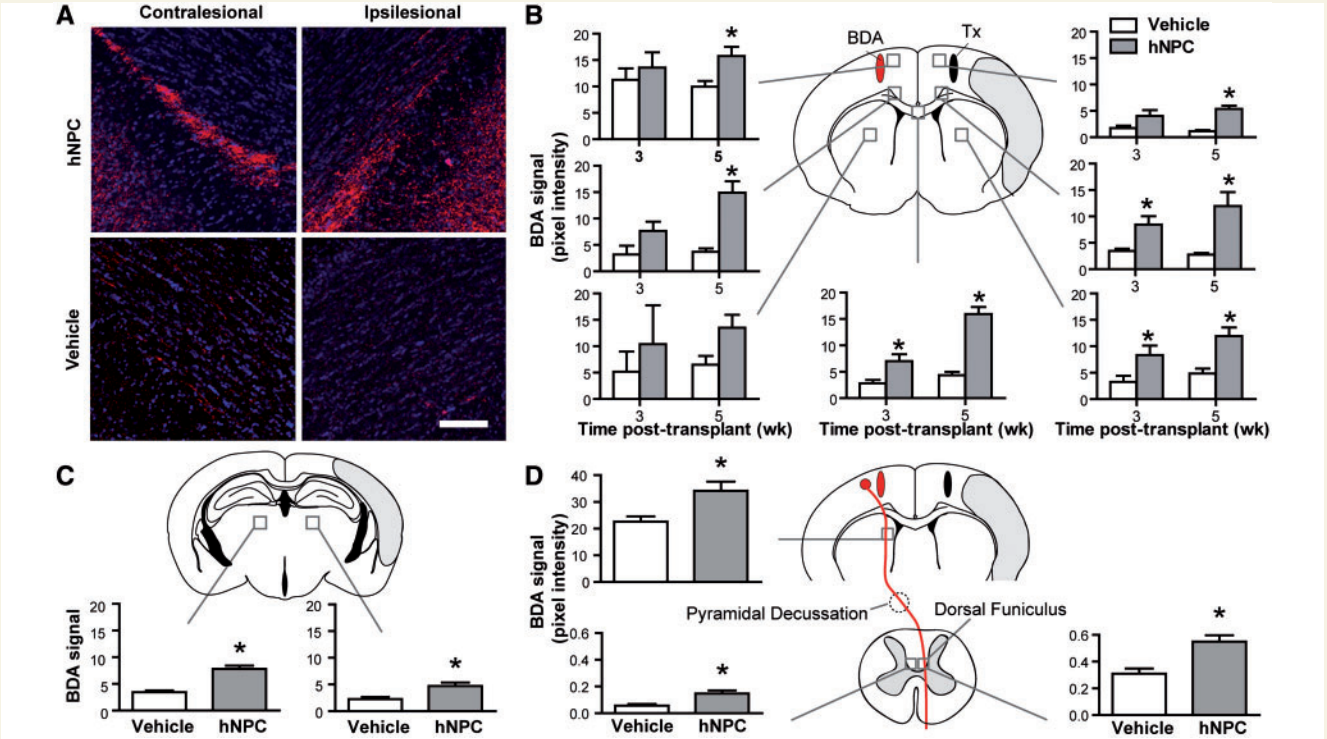

Figure 3 Human NPCs enhance axonal sprouting post-stroke. (**A**) Representative confocal images of biotinylated dextran amine (BDA) staining in the contra- and ipsilateral corpus callosum of human NPC- and vehicle-treated animals at 5 weeks post-transplantation. Scale bar = 100 μm. (**B**) Human NPC-grafted rats have significantly increased BDA-labelled fibre density at 3 weeks ($n = 6$) and 5 weeks ($n = 12$) post-transplantation in the cortex, corpus callosum and the ipsilesional striatum. At 5 weeks post-transplantation human NPC-grafted rats also have increased BDA signal in (**C**) the ipsi- and contralesional thalamus and (**D**) corticospinal fibres in the contralesional internal capsule and both the contra- and ipsilesional dorsal funiculus in the cervical spinal cord, C5 level. $^*P < 0.05$ human NPC compared with vehicle at same time point. Red oval = BDA injection site; black oval = human NPC transplant site.

transport) after stroke than vehicle controls. Moreover, amyloid precursor protein accumulation in the corpus callosum showed an overall negative correlation with recovery in the whisker-paw (Spearman correlation coefficient $\rho = -0.689$; $P = 0.015$) and cylinder test (Spearman correlation coefficient $\rho = -0.739$; $P = 0.008$) when combining data from both vehicle and human NPC groups, suggesting that reducing amyloid precursor protein and improving axonal transport is important for recovery.

Effects of human neural precursor cells on axonal transport are mediated by vascular endothelial growth factor

The effect of human NPCs and human NPC-secreted factors on axonal transport was determined *in vitro* using a microfluidic platform with one chamber seeded with primary cortical neurons and the other with or without human NPCs (Fig. 5D). Anterograde transport of dextran-labelled vesicles in the axons of the cortical neurons was measured in the axonal compartment (Fig. 5E). Co-culture with human NPCs significantly increased the mean velocity of anterogradely transported vesicles (Fig. 5F and Supplementary Fig. 5). Neutralization of VEGF, but not thrombospondins 1, 2, SPARC or Slit, inhibited the human NPC-mediated effect (Fig. 5F). Isotype control antibodies had no effect, and neutralization of these factors had no effect in vehicle-treated wells.

Discussion

We show that human NPC transplantation at 1 week after stroke significantly improved functional recovery in several behaviour tests and that this recovery correlated with human NPC-enhanced changes in dendritic and axonal structural plasticity. Human NPCs not only elicited these plasticity changes in the injured hemisphere but also helped recruit the uninjured hemisphere. Moreover, we demonstrate for the first time that human NPC transplantation enhanced recovery of stroke-impaired axonal transport. This is significant given the vital role of axonal transport for neuron function, survival and plasticity. Using co-culture assays, we could mimic these human NPC-induced effects on plasticity and transport *in vitro*, and through neutralization studies we identified four secreted factors that mediate these human NPC-induced effects. These data suggest that transplanted human NPCs enhance post-stroke recovery by secretion of factors that enhance endogenous brain repair mechanisms induced after stroke injury.

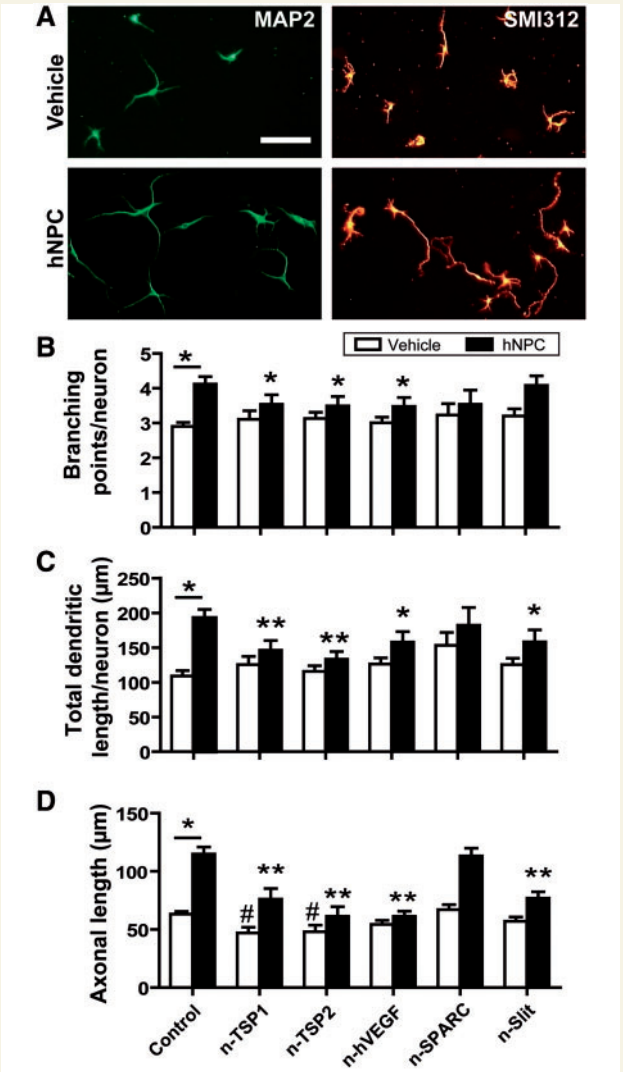

Figure 4 Identification of secreted factors mediating neurite plasticity *in vitro*. (**A**) Representative images of cortical neurons stained for microtubule-associated protein 2 (MAP2, labels dendrites) and SMI312 (labels axons) co-cultured without (vehicle) and with human NPCs. Scale = 50 µm. (**B–D**) Human NPCs significantly promoted dendritic branching (**B**), total dendritic length (**C**) and axonal outgrowth (**D**) of the cortical neurons; $^{*}P < 0.05$ 'control human NPC' compared with 'control vehicle'. Neutralization of the secreted factors with antibodies or the soluble slit receptor Roundabout-Fc significantly reduced the effects of human NPCs on these parameters as indicated. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 'control human NPC'; $^{\#}P < 0.05$ compared with 'control vehicle'. TSP = thrombospondins.

Table 1 Detection of human genes in human NPC-transplanted rat tissue

Human gene	Human NPCs		Vehicle	
	Average threshold cycle	±SD	Average threshold cycle	±SD
GAPDH	**26.4**	2.0	39.2	3.9
B2M	**26.0**	1.9	40.9	4.8
VEGFA	**33.8**	1.8	>45	–
Thrombospondin 1	**33.0**	2.5	36.0	2.1
Thrombospondin 2	**34.2**	2.3	>45	–
SPARC	**28.7**	3.1	>45	–
SLIT1	36.7	1.9	39.0	1.4
SLIT2	40.1	1.9	43.7	0.9
SLIT3	33.7	1.4	35.0	1.2

Expression of human reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2-microglobulin (B2M) and target genes was determined by quantitative polymerase chain reaction 7 days after human NPC or vehicle transplantation into ischaemic animals ($n = 4$ per group). Genes were considered positive for human-specific expression (indicated in bold) if the average threshold cycle was <35 in human NPC-transplanted tissue and >35 in vehicle-transplanted tissue. Individual data points are displayed in Supplementary Fig. 3.

Human neural precursor cells enhance the innate repair capacity of the brain

There is compelling evidence from both patient and animal data that the brain undergoes reorganization and rewiring of surviving circuits after ischaemia and this is postulated to underlie the spontaneous recovery observed after stroke (Dancause, 2006; Murphy and Corbett, 2009; Benowitz and Carmichael, 2010). Such restorative neuronal plasticity changes include an increase in afferent and efferent connections in both ipsi- and contralesional brain regions, resulting in part from changes in dendritic and axonal sprouting of surviving neurons.

Chronic changes in dendritic structural plasticity after stroke have been reported with increased contralesional layer V dendritic branching peaking at 18 days post-stroke (Jones and Schallert, 1992), while ipsilesional layer III branching was decreased (compared with uninjured animals) at 9 weeks post-stroke (Gonzalez and Kolb, 2003). Our data show for the first time that at 3 weeks post-stroke, human NPCs enhance dendritic length and arborization in layer V cortical neurons in both the ipsi- and contralesional cortex (despite the latter being remote from the site of transplantation), and this coincided with the onset of human NPC-induced functional recovery. Enhanced contralesional dendritic plasticity after stroke is thought to be due to compensatory increased use of the unimpaired limb (Jones and Schallert, 1994). However, this is not apparent in the human NPC-treated animals; despite enhanced contralesional plasticity they exhibit less relative use of the unimpaired limb than vehicle controls. Thus, the significance of human NPC-enhanced contralesional dendritic plasticity and its importance for early recovery remains to be determined. Of note, vehicle injection was detrimental to dendritic plasticity at 2 weeks post-injection with recovery by 4 weeks. The human NPCs appeared to prevent this injection injury, suggesting neuroprotective activity, be it direct or indirect. This raises the question whether this neuroprotective effect is a significant component of human NPC-induced recovery in the first few weeks following transplantation.

Human NPC-induced dendritic changes in the contralesional cortex were transient returning to baseline by 5 weeks post-stroke, thus indicating that human NPCs could not prevent the contralesional branch regression previously reported (Jones and Schallert,

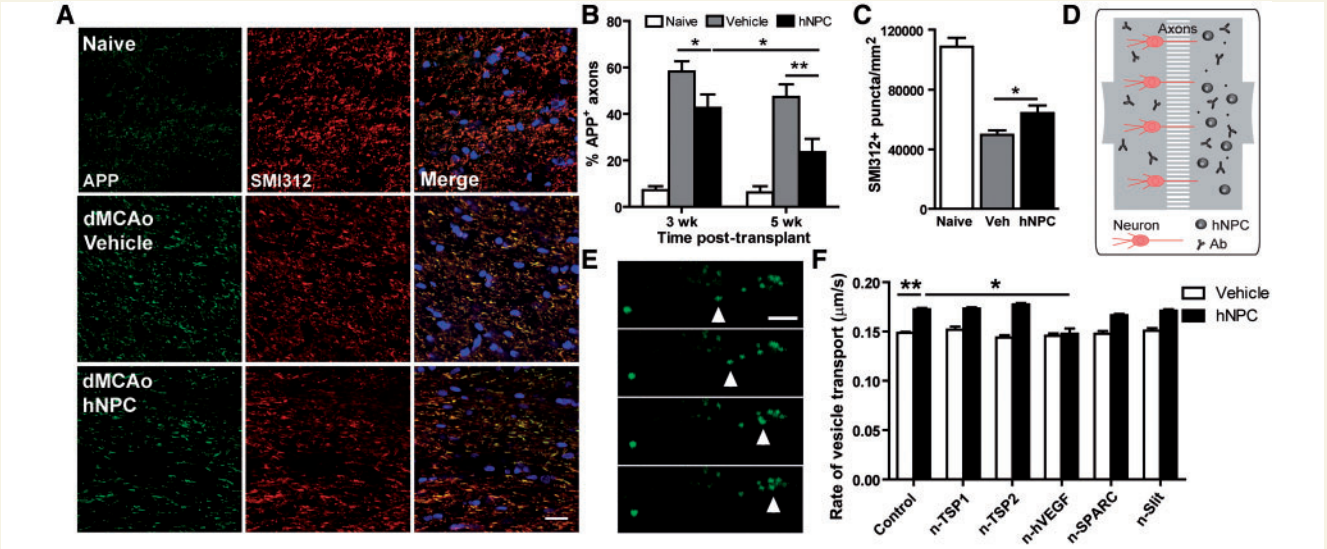

Figure 5 Effects of human NPCs on anterograde axonal transport *in vivo* and *in vitro*. (**A**) Representative confocal images of amyloid precursor protein (APP) and SMI312 staining of the corpus callosum at 6 weeks post-stroke (5 weeks post-transplant) in uninjured (naïve), vehicle treated (dMCAo, vehicle) and human NPC-grafted (dMCAo, hNPC) animals. Scale = 20 μm (**B**) Quantification of the percentage of amyloid precursor protein-positive SMI312 puncta to determine the percentage of amyloid precursor protein-positive axons; 3 weeks, $n = 6$; 5 weeks $n = 12$. (**C**) Quantification of total axonal density at 6 weeks post-stroke ($n = 12$). (**D**) Schematic of microfluidic assay system for analysis of axonal transport *in vitro*. (**E**) Time-lapse confocal images of dextran-labelled vesicles used to measure the rate of axonal transport. Arrowhead indicates the same vesicle at different times. Scale = 5 μm. (**F**) Human NPCs enhance the rate of axonal transport in cortical neurons compared with vehicle treatment. Neutralization of VEGF, but not other factors significantly reduced this human NPC-mediated effect. $*P < 0.05$, $**P < 0.01$. dMCAo = distal middle cerebral artery occlusion.

1992), and that contralesional dendritic changes are not necessary for continued recovery at later time points. In contrast, ipsilesional changes (i.e. in the human NPC-grafted hemisphere) were maintained at 1 month post-transplantation with no sign of abating, suggesting that local effects of the human NPCs are necessary to maintain dendritic changes. The biggest change in dendritic structural plasticity was observed in basilar dendrites where the majority of synaptic inputs are found (Larkman, 1991); however, the significance of this for neuron function remains to be elucidated (Nevian *et al.*, 2007; Spruston, 2008). The pattern of early dendritic changes in the contralesional cortex followed by a switch to more dominant changes in the ipsilesional cortex at later times is reminiscent of brain remapping results in patients and animals. These remapping studies show that stimulation of the injured limb early after stroke recruits the contralesional cortex and this switches back to the ipsilesional cortex at later time points (Dancause, 2006; Benowitz and Carmichael, 2010). It would thus be of interest to determine the significance of these dendritic changes to such remapping data.

Axonal sprouting occurs after stroke with new projections thought to target areas denervated by the stroke injury (Benowitz and Carmichael, 2010). In rodent and primate models of ischaemic cortical injury, such sprouting has been observed locally around the infarct area (Carmichael *et al.*, 2001; Conner *et al.*, 2005; Dancause, 2006; Li *et al.*, 2010), and interhemispheric axonal outgrowth from the intact cortex to the injured hemisphere has also been observed (Carmichael, 2008). Our BDA axonal tracer data suggest that human NPC transplantation promotes this interhemispheric cortical sprouting in corticocortical, corticostriatal and corticothalamic pathways. These data corroborate previous reports using human umbilical cord blood cells (Xiao *et al.*, 2005) and human embryonic-derived neural progenitor cells (Daadi *et al.*, 2010) in immunosuppressed or immunocompetent animals, suggesting that this phenomenon is independent of stem cell type used and T cell status of the host. Consistent with enhanced axonal sprouting, human NPCs also enhanced expression of the axonal growth cone protein GAP-43 in the same regions. However, GAP-43 is not purely a marker of regenerating axons as it is also expressed on non-neuronal cells such as astrocytes and oligodendrocytes (Carmichael, 2008); the importance of this for regeneration is not understood.

Human NPC transplantation also enhanced stroke-induced remodelling of cortical–spinal tract axons originating from the contralesional cortex (i.e. intact corticospinal tract). The fact that the number of BDA-labelled fibres not only increased in the ipsilateral dorsal funiculus (intact corticospinal tract) but also in the contralateral dorsal funiculus (injured corticospinal tract) implies that human NPCs may enhance collateral (cross midline) sprouting of the intact corticospinal tract into denervated regions of the spinal cord (Chen *et al.*, 2002; Liu *et al.*, 2008). Marrow stromal cell treatment enhanced this collateral sprouting (Liu *et al.*, 2008), but unlike human NPC treatment, marrow stromal cells had no effect on fibre density in the intact corticospinal tract (Liu *et al.*, 2008). The human NPC-induced changes in both corticospinal tract and transcallosal axonal sprouting statistically correlated with human NPC-enhanced functional recovery; a similar correlation was

found between recovery and marrow stromal cell-induced corticospinal tract sprouting (Liu *et al.*, 2008, 2011). Together, these data imply that human NPC-induced axonal plasticity is an important mechanism for human NPC-induced recovery, although a direct causal link remains to be demonstrated.

A key finding of our study is the ability of human NPCs to enhance recovery of axonal transport after stroke. This has major implications for recovery of white matter injury. Axonal transport is disrupted after ischaemia (Kataoka *et al.*, 1989; Valeriani *et al.*, 2000; Wakita *et al.*, 2002) and is indicative of axonal damage. Amyloid precursor protein is commonly used as a marker of impaired transport and axonal degeneration (Stone *et al.*, 2000; Valeriani *et al.*, 2000) as it is constitutively expressed in neurons and normally subject to fast axonal transport (Koo *et al.*, 1990). However, under pathological conditions, amyloid precursor protein accumulates in axons, which can be both a consequence and a cause of disrupted transport (Stone *et al.*, 2000; Gunawardena and Goldstein, 2001; Salehi *et al.*, 2006). Our results confirm that stroke induces amyloid precursor protein accumulation in the corpus callosum and it remains elevated at 6 weeks post-stroke, implying extended perturbation of axonal transport. Human NPC transplantation reduced amyloid precursor protein accumulation and accelerated the decrease of amyloid precursor protein over time, which strongly suggests that human NPCs enhance recovery of fast axonal transport after stroke. Furthermore, by direct measurement of vesicle transport in cultured cortical neurons, we confirmed that human NPCs can enhance axonal transport. This is a significant finding as axonal transport is fundamental to neuron function, not only for proper functioning and survival of existing axons but also for plasticity changes such as axonal sprouting and synaptogenesis. Therefore, human NPC-induced restoration of impaired axonal transport after stroke may not only enhance the function of existing fibre tracts but may also be a key upstream event of human NPC-induced structural plasticity.

Given that axonal tracers-like BDA undergo fast axonal transport, this questions the interpretation of tract tracing data; is increased axonal labelling really indicative of a greater number of axons or does it merely reflect increased axonal transport? Because axonal sprouting, transport and survival are somewhat co-dependent, it probably represents a combination of events. Consistent with this, human NPCs enhanced both axonal transport and neurite outgrowth (measured directly) *in vitro*. Regardless of mechanism, increased BDA labelling probably signifies enhanced fibre tract integrity *in vivo* whether this is due to increased numbers of axons or better functioning (transport) of axons. The reduced atrophy of the corpus callosum observed in human NPC-treated animals provides additional indirect evidence for improved fibre tract integrity.

Identification of putative secreted factors involved in human neural precursor cell-mediated plasticity

The majority of the human NPCs remained in an immature state at 5 weeks post-stroke, therefore, differentiation into functionally mature cells is not necessary for human NPC-induced recovery. Instead, it is postulated that transplanted human NPCs elicit their effects through secretion of relevant factors. Using *in vitro* co-culture systems in which human NPCs mimicked the effects observed *in vivo*, we identified secreted molecules putatively involved in human NPC-induced axonal transport and neurite sprouting. We found that thrombospondins 1 and 2, VEGF, Slit, but not SPARC, were important for human NPC-induced dendritic and axonal outgrowth. SPARC may require other cells (e.g. oligodendrocytes) to potentiate its effects on neurite growth (Au *et al.*, 2007). Human NPCs also increased neuronal survival; whether this is related to their effects on neurite outgrowth is unclear. However, several mechanisms may be involved as depletion of thrombospondin 2 and Slit only affected neurite outgrowth without affecting cell survival. Only VEGF was important for human NPC-enhanced axonal transport. VEGF has previously been reported to be transported by axons (Storkebaum *et al.*, 2005), whether this is important for its effects on axonal transport remains to be determined.

Because most antibodies used in these experiments may bind both human and rodent forms of the protein, further studies are required to determine whether direct secretion of these factors by the human NPCs or human NPC-induced secretion of these factors by the primary rat cultures is important for the observed effects. However, neutralization of VEGF was achieved using Avastin, which binds human but not rodent VEGF (Ferrara *et al.*, 2004), strongly implying that direct secretion of VEGF by human NPCs plays a significant role. Furthermore, using a small interfering RNA knockdown approach, Yu *et al*. (2008) reported that thrombospondin 1 was important for marrow stromal cell-induced neurite outgrowth in retinal ganglion cell cultures, thus strengthening the idea that direct secretion of thrombospondin by human NPCs is involved.

Whether these factors are also important for the *in vivo* effects of human NPCs remains to be determined but at least many of them are expressed by the human NPCs in the stroke brain. We have previously reported the importance of stem cell-secreted VEGF for human NPC-induced functional recovery (Horie *et al*., 2011). Thrombospondins 1 and 2 are also strong candidates since growing evidence suggests the importance of thrombospondins after stroke as they are upregulated after cerebral ischaemia (Lin *et al.*, 2003), and we recently reported that thrombospondins 1 and 2 knockout mice have reduced functional recovery and axonal sprouting after stroke (Liauw *et al.*, 2008). Thus, these human NPC-secreted factors warrant further investigation, but clearly other modulatory factors will also be involved (Carmichael, 2008).

Translational implications

Human NPCs have clinical potential for stroke therapy and we (Svendsen) have a clinical grade bank of foetal-derived human NPCs, which makes this a realistic goal. Understanding the mode of action of human NPCs in the post-ischaemic brain will be important for the successful translation of cell transplantation strategies to the clinic. For example, if modulation of host brain plasticity is a major human NPC mechanism of action, this could

dictate the best time to transplant cells after stroke; 'network re-learning' occurs within weeks of stroke and continues for several months, making it a good therapeutic target with a large time window of intervention. Furthermore, knowing what changes the human NPCs elicit in the brain offers useful surrogate indicators of transplanted cell activity. For example, changes in fibre tract integrity can be monitored non-invasively with diffusion tensor MRI, which can evaluate cell therapy efficacy and also highlight potential side effects resulting from too much or inappropriate human NPC-induced plasticity. Moving from the bench to the clinic also raises cell manufacturing issues, in particular designing bioassays to predict clinical efficacy of the cell product. Our data imply that human NPC-induced changes in neurite sprouting and axonal transport would be useful predictors of cell efficacy. Therefore, the *in vitro* assays we developed here to test these parameters, with their potential for high throughput, could serve as useful bioassays. Finally, the translational feasibility of human NPC therapy will depend on many variables including the difference in scale between the rodent and human brain which may affect the ability of human NPCs to induce effective plasticity. Furthermore, patients experience a rehabilitative or enriched post-stroke environment compared to laboratory animals, which *per se* is reported to enhance plasticity and recovery in rodents (Biernaskie and Corbett, 2001; Wurm *et al.*, 2007). Therefore, demonstrating that human NPCs can further enhance recovery in rats under enriched conditions will be critical (Mattsson *et al.*, 1997; Hicks *et al.*, 2007). These issues warrant further investigation as cell therapies move to the clinics.

Conclusion

In summary, we have demonstrated that transplanted human NPCs enhance axonal transport, dendritic branching and axonal sprouting of host neurons after stroke, and that these plasticity changes correlated with human NPC-induced recovery. Furthermore, we identified several secreted factors that mediated these same changes *in vitro*. Therefore, we postulate that transplanted human NPCs aid in recovery through secretion of factors that enhance the natural brain remapping that occurs following stroke. Such a mechanism of action offers a large therapeutic window for post-stroke stem cell intervention.

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Supplementary material

Supplementary material is available at *Brain* online.

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