MRI visualization of endogenous neural progenitor cell migration along the RMS in the adult mouse brain: Validation of various MPIO labeling strategies

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A B S T R A C T

The adult rodent brain contains neural progenitor cells (NPCs), generated in the subventricular zone (SVZ), which migrate along the rostral migratory stream (RMS) towards the olfactory bulb (OB) where they differentiate into neurons. The aim of this study was to visualize endogenous NPC migration along the RMS with magnetic resonance imaging (MRI) in adult healthy mice. We evaluated various in situ (in vivo) labeling approaches using micron-sized iron oxide particles (MPIOs) on their efficiency to label endogenous NPCs. In situ labeling and visualization of migrating NPCs were analyzed by a longitudinal MRI study and validated with histology. Here, we visualized endogenous NPC migration in the mouse brain by in vivo MRI and demonstrated accumulation of MPIO-labeled NPCs in the OB over time with ex vivo MRI. Furthermore, we investigated the influence of in situ injection of MPIOs on adult neurogenesis. Quantitative analysis of bromodeoxyuridine labeled cells revealed altered proliferation in the SVZ and NPC migration after in situ MPIO injection. From the labeling strategies presented in this report, intraventricular injection of a small number of MPIOs combined with the transfection agent poly-l-lysine hydrobromide was the best method as labeling of the NPCs was successful and proliferation in the SVZ was only marginally affected. While MRI visualization of endogenous NPC migration can provide insight into aberrant NPC migration in disease models, this work emphasizes the importance to carefully explore the impact on adult neurogenesis when new in situ labeling strategies are developed.

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Introduction

The discovery of neural progenitor cells (NPCs) in the adult brain raised hope for new treatment strategies to repair damage of the central nervous system. In the rodent brain, the rostral migratory stream (RMS) contains NPCs, generated in the subventricular zone (SVZ), which migrate towards the olfactory bulb (OB) where they differentiate into neurons (for review see Alvarez-Buylla and Garcia-Verdugo, 2002). Recent studies have shown that NPCs, arising from the SVZ, can also migrate towards damaged brain regions and subsequently differentiate into the phenotype of the destroyed cells (Arvidsson et al., 2002; Picard-Riera et al., 2002). These studies indicate that the adult brain has the capacity for neuronal self-repair. Disturbed adult neurogenesis has been reported in neurodegenerative diseases (Curtis et al., 2007). An increase in cell proliferation in the SVZ has been observed in Alzheimer patients, as well as in a mouse model for Alzheimer’s disease (Jin et al., 2004). Conversely, in a 6-hydroxydopamine rat model for Parkinson’s disease, experimental depletion of dopamine resulted in decreased cell proliferation in the SVZ (Baker et al., 2004). This reduction was also demonstrated in human Parkinson’s disease (Höglinger et al., 2004). The latter results highlight the importance of a molecular mechanism which preserves the microenvironment for normal neurogenesis, and hence the capacity for self-repair in the adult brain. Direct manipulation of endogenous NPCs is currently considered a very promising line of investigation for the development of new cell replacement therapies for several neurodegenerative diseases.

Undoubtedly, in vivo visualization of cell migration is the ultimate tool in the follow-up of deviating NPC migration patterns and potential cell therapies. Currently, most efforts incorporate magnetic resonance imaging (MRI) because of its superior spatial resolution, allowing in vivo monitoring of MR contrast-labeled stem cell recruitment with high accuracy. A commonly used procedure for in vivo visualization of cell migration has been the transplantation of

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stem cells, labeled in culture with superparamagnetic iron oxide particles (Hoehn et al., 2002; Jendelova et al., 2003; Kraitchman et al., 2003; Lee et al., 2004; Modo et al., 2004; Shapiro et al., 2006b). Recently, a report has provided evidence that endogenous NPCs in the SVZ could be labeled using micron-sized iron oxide particles (MPIOs) by in situ injection of the MPIOs into the lateral ventricle (LV) near the SVZ of adult rats (Shapiro et al., 2006a; Sumner et al., 2009). MPIO-labeled cells could be distinguished from the surrounding tissue as they appear as hypointense (dark) spots on $T_2$-weighted MR images. The advantages of MPIOs in labeling cells for MRI visualization have been listed and demonstrated in various *in vitro* and *in vivo* experiments over the last few years (Shapiro et al., 2004, 2006a; Sumner et al., 2009). Nevertheless, the strategy of *in situ* labeling with MPIOs suffers from the limitation that a large quantity of the particles needs to be injected, resulting in a large degree of signal inhomogeneity (loss of signal intensity) and image distortion on the MR images at the site of injection. The first effort on reducing the injection volume for *in situ* labeling of NPC has been currently performed in adult rats (Panizzo et al., 2005). This study showed that labeling of endogenous NPC after intraventricular injection of Endorem in a small volume was only successful after complex formation with the transfection agent protamine sulfate. Besides the limitation of MPIOs on MR images, it was recently reported that *in situ* MPIO labeling resulted in a rather low NPC labeling efficiency (Sumner et al., 2009), which urges further investigations on the impact of *in situ* labeling on adult neurogenesis.

In view of future applications in mouse models for various neurodegenerative diseases, our purpose was to visualize endogenous NPC migration along the RMS to the OB with MRI in healthy adult mice. In an attempt to optimize *in situ* labeling with MPIOs, we examined five *in situ* labeling strategies and analyzed the impact of *in situ* MPIO injection on adult neurogenesis.

### Materials and methods

**Experimental design**

The work in this report consists of two independent studies: (1) a longitudinal MRI study to visualize endogenous NPC migration and (2) study on the impact of *in situ* MPIO injection on adult neurogenesis.

### Longitudinal MRI

Mice were divided into six experimental groups (see Table 1). Mice of the control group (*n* = 4) did not receive a stereotactic injection. The other five groups represent different *in situ* labeling strategies. For the first labeling strategy, mice (*n* = 7) were stereotactically injected with 10 μl MPIOs (2.7 × 10^7^ particles; 3.00 mg Fe/ml) in the LV. Next three groups of mice received a stereotactic injection in the LV with a smaller amount of particles (9.1 × 10^5^ particles): 1.5 μl MPIOs (0.67 mg Fe/ml; *n* = 6), 1.5 μl MPIOs-PLL complex (0.67 mg Fe/ml; *n* = 4) or 1.5 μl MPIOs-FUG complex (0.67 mg Fe/ml; *n* = 4). The last group of mice (*n* = 7) was injected near the RMS with 1.5 μl MPIOs (9.1 × 10^5^ particles; 0.67 mg Fe/ml). *In vivo* MR imaging was performed at 3, 5 and 8 weeks post-injection (PI). After each time point of *in vivo* MRI, at least one animal of each group was sacrificed for *ex vivo* MRI and immunohistochemistry. In addition, after the last MRI scan (8 weeks PI), one mouse of each group was sacrificed for electron microscopic examination.

### Impact of *in situ* MPIO injection on adult neurogenesis

Depending on the site of injection, in the LV or near the RMS, we examined the impact of MPIO injection on either cell proliferation in the SVZ or NPC migration (see Table 2). To label dividing cells in the brain, the DNA synthesis marker 5-bromo-2′-deoxyuridine (BrDU; Sigma-Aldrich, Bornem, Belgium) was dissolved in phosphate-buffered saline (PBS) and administered intraperitoneally at a dose of 50 mg/kg body weight.

For the effect on cell proliferation in the SVZ, two time points were investigated. Firstly, to detect the instant effect of surgical injection and the presence of MPIOs in the LV, mice were sacrificed 1 day after intraventricular injection. One single dose of BrDU was given to the mice 3 h prior to perfusion to assess proliferation. Three experimental groups were analyzed: controls (*n* = 3), 1.5 μl PBS injection (sham-operation; *n* = 4) and 1.5 μl MPIOs injection (9.1 × 10^5^ particles; *n* = 4). Secondly, to detect the long-term effect, i.e. impact of the prolonged presence of MPIOs in the LV, animals were sacrificed at 3 weeks PI. Mice were also treated with one single dose of BrDU 3 h prior to perfusion. BrDU quantification was performed on four groups of mice: without an injection (*n* = 3), with a 10 μl MPIOs injection (2.7 × 10^5^ particles; *n* = 4), with a 1.5 μl MPIOs injection (9.1 × 10^5^ particles; *n* = 4) and with a 1.5 μl MPIOs-PLL injection (9.1 × 10^5^ particles; *n* = 3). In addition, MHC-II histology was carried out on the groups of the long-term effect to analyze a possible inflammatory reaction after intraventricular MPIO injection.

For the effect on cell migration, mice were treated with one single dose of BrDU 1 day before injection near the RMS and were sacrificed 3 weeks PI to ensure BrDU-labeled cells reached the OB within this time period. We compared the number of BrDU-labeled cells in the left and right hemisphere of the OB.

### Table 1

<table>
<thead>
<tr>
<th>No injection</th>
<th>10 μl LV</th>
<th>1.5 μl LV</th>
<th>1.5 μl RMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>n = 4</td>
<td>n = 4 + 3</td>
<td>n = 4 + 3</td>
</tr>
<tr>
<td>MPIOs</td>
<td></td>
<td>n = 4 + 2</td>
<td>n = 4 + 3</td>
</tr>
<tr>
<td>MPIOs-PLL</td>
<td></td>
<td>n = 4 + 0</td>
<td>n = 4 + 3</td>
</tr>
<tr>
<td>MPIOs-FUG</td>
<td></td>
<td>n = 0</td>
<td>n = 4</td>
</tr>
</tbody>
</table>

MPIOs, micron-sized iron oxide particles; MPIOs-PLL, micron-sized iron oxide particles combined with the transfection agent poly-l-lysine; MPIOs-FUG, micron-sized iron oxide particles combined with the transfection agent Fugene-6; LV, lateral ventricle; RMS, rostral migratory stream. (*n*) indicates the number of animals injected and scanned for each condition. Initially we started with four animals for each condition. To test the reproducibility of a successful strategy (see Table 3), we added three animals to these groups. One animal of the 1.5 μl MPIOs in the LV was left out of the study as the injection coordinates were not accurate. For the three additional MPIOs-PLL injections, the MPIOs were mixed with the stock solution (1.5 mg PLL/ml) instead of the 0.3 mg PLL/ml. These animals were also excluded from the study as it has been reported that a high concentration of PLL may result in cytotoxicity (Symonds et al., 2005).

### Table 2

<table>
<thead>
<tr>
<th>SVZ proliferation</th>
<th>NPC migration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 weeks PI</td>
</tr>
<tr>
<td>Instant effect</td>
<td>Long-term effect</td>
</tr>
<tr>
<td>1 day PI</td>
<td>3 weeks PI</td>
</tr>
<tr>
<td>No injection</td>
<td>1.5 μl LV</td>
</tr>
<tr>
<td>Controls</td>
<td><em>n</em> = 3</td>
</tr>
<tr>
<td>MPIOs</td>
<td><em>n</em> = 4</td>
</tr>
<tr>
<td>MPIOs-PLL</td>
<td><em>n</em> = 4</td>
</tr>
<tr>
<td>MPIOs-FUG</td>
<td><em>n</em> = 4</td>
</tr>
<tr>
<td>PBS (Sham)</td>
<td><em>n</em> = 4</td>
</tr>
</tbody>
</table>

PBS, phosphate-buffered saline; MPIOs, micron-sized iron oxide particles; MPIOs-PLL, micron-sized iron oxide particles combined with the transfection agent poly-l-lysine; PI, post-injection; SVZ, subventricular zone; NPC, neural progenitor cell; LV, lateral ventricle; RMS, rostral migratory stream. (*n*) indicates the number of animals used for the BrDU quantification for each condition. For the instant and long-term effect on the SVZ proliferation, four animals were injected for each injection strategy. For the NPC migration, five animals per injection strategy were injected. Some animals were excluded from the study during the histological procedure when sectioning or staining was not properly performed.
right OB of mice injected with 1.5 μl PBS (sham-operation, n = 5) with mice injected with 1.5 μl MPIOs (n = 3).

In the last 3 days prior to perfusion, in vivo MRI was performed on all animals of the 3 weeks PI time point to verify the results of the longitudinal MRI study.

**Animals**

Male adult C57BL/6j mice (Charles River Laboratories, L’Arbresle Cedex, France) were used for both studies. All aspects of animal experiments were carried out in compliance with national and European regulations and were approved by the Animal Care and Use Committees of the University of Antwerp.

**MPIO preparation and surgical injection**

As a MR contrast agent, we used a 1.63 μm diameter polystyrene/divinylbenzene-coated fluorescent iron oxide particles (MPIOs, 3.00 mg Fe/ml, green fluorescent dye: 480 nm excitation, 520 nm emission; Bangs Laboratories, Fishers, IN, USA). Two injection volumes containing different numbers of particles were used: a volume of 10 μl with 2.7 × 10⁷ particles (3.00 mg Fe/ml) and a volume of 1.5 μl with 9.1 × 10⁶ particles (0.67 mg Fe/ml). Two non-viral transfection agents were used in this study: poly-l-lysine hydrobromide (PLL, MW >300 kDa, Sigma-Aldrich, Munich, Germany), a cationic polyamine, and Fugene-6 (FUG, Roche, Almere, The Netherlands), a multi-component lipid-based (non-liposomal) reagent. Both compounds are commercially available and have been previously used to improve intracellular labeling with MR contrast agents (Frank et al., 2003; Hoehn et al., 2002; Kalish et al., 2003). A PLL stock solution of 1.5 mg/ml was prepared and stored at 5 °C. Prior to injection, the stock solution was further diluted to 0.3 mg/ml and mixed with the MPIOs (3.0 mg Fe/ml), yielding a final MPIO concentration of 0.67 mg Fe/ml with 0.045 mg PLL/ml. FUG was diluted 1:1000, mixed with the additional 5 min and then slowly withdrawn. After surgery, 0.50 mL of 0.9% sodium chloride solution was injected to the transfection agent to hybridize with the iron oxide particles. All dilutions were performed with 0.9% sodium chloride solution. For sham injections we used 0.01 M PBS.

The animals were anesthetized intraperitoneally with ketamine (Anesketin: 100 mg/ml, Eurovet NV/SA, Heusden-Zolder, Belgium) and medetomidine (Domitor: 1 mg/ml; Pfizer Animal Health s.a., Louvain-la-Neuve, Belgium), and positioned in a stereotactic head frame. Stereotactic coordinates (relative to bregma) were as follows: for the LV injection: anterior 0.3 mm–lateral 1 mm–dorsal 2.3 mm; for the RMS injection: anterior 1.7 mm–lateral 0.7 mm–dorsal 4.1 mm. A Hamilton syringe (26 G Gauge, VWV International, Haarsoede, Belgium) was connected with a pump to inject at a constant rate of 0.50 μl/min. After injection, the needle was left in place for an additional 5 min and then slowly withdrawn. After surgery, anesthesia was reversed using atipamezol (Antisedan 5 mg/ml, Pfizer Animal Health s.a.), which was administered intraperitoneally.

**Magnetic resonance imaging**

MRI experiments were performed at 300 MHz either on a 7-T magnet from MagneX Scientific (Oxfordshire, UK) with a console from MR Solutions (Guildford, UK) or a 7-T PharmaScan system (Bruker, Ettlingen, Germany). A Helmholtz volume transmit antenna (30 mm) and actively decoupled surface receiving antenna (20 mm) were used. The mice were anesthetized by inhalation of 3% isoflurane (Isoflo®) for induction and 1% isoflurane for maintenance in a mixture of 30% O₂ and 70% N₂ at a flow rate of 600 ml/min. Breathing rate and body temperature were continuously monitored with pcam software (SA Instruments rents, NY, USA). The monitoring system allowed us to maintain the breathing rate at 110±10 breaths/min and the body temperature within a narrow range of 37±0.5 °C. First, sagittal, coronal and transversal images were obtained to allow proper slice orientation of the subsequent high-resolution (isotropic resolution of 75 μm) 3D T₁-w–weighted GE sequence (matrix = 128³, FOV = 9.6 mm covering the forebrain and the OBs, TR = 40 ms, TE = 13 or 8 ms, 30° flip angle and six repetitions with a total scanning time of approximately 65 min). To prevent phase wrapping due to the small FOV, two saturation slices (sagittal/oblique orientation, thickness = 5 mm) were positioned on each side of the brain. Animals were sacrificed with an intraperitoneal overdose of pentobarbital (Nembutal; CEVA Santé Animale, Brussels, Belgium) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4) doped with 1 mM Gd-DOTA. The whole mouse head was fixed overnight in 4% PFA at 4 °C prior to transfer to PBS. The fixed mouse head was covered with Parafilm® and placed in the stereotactic device in the same orientation as a living mouse. High-resolution 3D GE images were obtained with the following image parameters: TR = 100 ms, TE = 6.3 ms, FOV = 17 * 17 * 17 mm (covering the whole mouse brain), matrix = 256 * 256 * 256 (66 μm isotropic voxel size), 30° flip angle and six averages, with a total scanning time of approximately 10 h 56 min.

**Post processing of MR images**

For post processing, Matlab software (version 7.1, The Mathworks Inc., Massachusetts, USA) and Amira software (version 4.0.1, Mercury Computers Systems, CA, USA) were used.

Custom-written Matlab software was used to extract the six repetitions acquired in vivo with the high-resolution 3D T₁-w–weighted GE sequence. Subsequently, with Amira, we inspected each repetition data set to verify data quality. When a data set with movement artifacts was present, we discarded it from further processing. The selected repetition data sets were affine registered and transformed to the data of the first repetition. Finally, a mean of the co-registered 3D data was calculated.

To compare the success rate of labeled NPC migration for the different injection strategies, we estimated the number of voxels in the OB representing hypointense spots on 3D ex vivo MR images. The volume of dark spots in the core of the OBs was estimated by counting the number of voxels displaying signal intensity below a predefined threshold. We used a threshold value of mean - 3 * SD. This value was obtained from visual interpretation of different threshold values applied on an ex vivo MRI where NPC migration towards the OB was clearly distinguishable. Due to the use of a surface receiving antenna, a signal bias between the left and right OB occurred when the mouse head was not positioned straight under the antenna. As general debiasing (SPM 5 software, http://www.fil.ion.ucl.ac.uk/spm) of the MRI data did not work properly, we implemented a method for threshold adjustment based on the signal intensity of volumes of interest (VOIs). We manually outlined a segment in each OB covering 20 transversal slices. Subsequently, within each OB segment, a rectangular VOI was defined (sagittal selection: width = 0.66 mm (10 slices), ventral–dorsal selection: width was minimized to include brain tissue within the sagittal selection), covering the region where the RMS enters the OB (Fig. 1; VOI₁: core of the OB ipsilateral to MPIO injection; VOI₂: core of the OB contralateral to MPIO injection). The complementary selections of the OB, containing the outer layers, are VOI₁ (Fig. 1; outer layers of the ipsilateral OB) and VOI₂ (Fig. 1; outer layers of the contralateral OB). Mean and standard deviation of the VOI signal intensities were measured for calculation of the threshold values to use for segmentation of dark spots. For the contralateral side (VOI₂) the applied segmentation threshold was: mean VOI₂ – 3 * SD VOI₂. In order to define the segmentation threshold for the ipsilateral side (VOI₁), the percentage of signal difference (due to bias field)
between the outer layers of the two bulbs (VOI\(_1\) and VOI\(_2\)) was calculated as follows: 
\[ \% = 100 - \left( \frac{\text{mean VOI}_3 \times \text{mean VOI}_4}{100} \right) \times 3 \times SD \text{ VOI}_4 \].

Minimum intensity projections (mIPs) were created from a subsample of sagittal slices of ex vivo 3D MRI covering the same sagittal selection (width = 0.66 μm) of the contrast quantification, i.e., the region where the RMS enters the OB.

**Immunohistochemistry**

Serial 50 μm coronal sections were made with a microtome (vibratome, St. Louis, MO, USA). Double immunofluorescence staining was performed overnight at room temperature in PBS-10% horse serum (v/v)–0.1% Triton (v/v) with the following antibodies: goat anti-Dcx as a marker for immature neurons (1:200; Chemicon International, Biotechnology, Heidelberg, Germany) and mouse anti-NeuN for labeling of mature neurons (diluted 1:200; Chemicon International, Heule, Belgium). The next day, sections were incubated with the fluorescently labeled secondary antibodies at room temperature. Endogenous peroxidase activity was blocked by 10 min incubation with H\(_2\)O\(_2\) (3% in PBS). Overnight incubation with the primary antibody (rat anti-BrdU, Accu-Specs/Labconsult, Brussels, Belgium) at 4 °C was followed by a 30-min incubation (at room temperature) with the biotinylated secondary antibody (donkey anti-rat, Dianova/Jackson ImmunoResearch, Hamburg, Germany) and a 30-min incubation with StreptABComplex-horseradish peroxidase (HRP) (Dako, Glostrup, Denmark). This biotin–streptavidin–peroxidase complex was visualized with 3.3-diaminobenzidine (DAB) (0.4 mg/ml, Sigma-Aldrich) and H\(_2\)O\(_2\) (0.03%, v/v).

**BrdU quantification and statistics**

A random-sampling stereological counting tool, the optical fractionator method, was used to count total numbers of BrdU+ cells. For the effect on the cell proliferation, BrdU+ cells were counted in the left and right SVZ. The area of interest was manually delineated as a small region covering the lateral wall of the LV in five sections, at 250 μm intervals throughout the LV. For the effect on cell migration, BrdU+ cells were counted in the left and right OB. The area of interest included the whole bulb on three sections at 250 μm intervals from the central part of the OB. A regular grid was superimposed over the area of interest and a three-dimensional counting frame of fixed size was placed within each square of the grid. In this setup, all cells were equally likely to be sampled. Cells within the counting frames were counted and estimations of the total number of cells (in the volume of interest representing the SVZ) and of the cell density (total number of cells per OB volume) were obtained with Stereoinvestigator software (version 5; MicroBrightField, Magdeburg, Germany) and a Leica DMR Biopoint 2 microscope (Leica Microsystems, Wetzlar, Germany).

The numbers of dividing cells as estimated by stereological quantification were statistically compared by using non-parametric tests appropriate for small populations (SPSS 14.0). Kruskal–Wallis tests were performed for multiple-group comparison, Mann–Whitney tests were used for two-group comparison. The results were considered significant when \( P < 0.05 \).

**MHC-II histology**

For MHC-II histology, serial 50 μm coronal sections were made with a microtome (vibratome, St. Louis). Sections were incubated with biotinylated rat anti-mouse MHC class II antigen (1:100 dilution, eBioscience, San Diego, USA) followed by incubation with StreptABComplex (Dako), visualization with DAB to detect antigen-presenting cells and counterstained with hematoxilin (Dako). Coronal sections were selected from the beginning of the LV up to the level of the hippocampus.
McDaniel et al., 2001

or FUG) to the MPIOs. Mice injected in the LV with 1.5 μMPIOs-FUG (C) showed dark spots at the level of the RMS and OB on the MR images. In an attempt to enhance uptake of the contrast particles by the cells of the ventricular wall, we added a transfection agent (PLL agent. Whereas a remarkably decreased signal inhomogeneity was observed at the site of injection on the MR images (Figs. 2A and B). An injection of 10 μl completely fills the LV with iron oxide particles (volume of the LV is less than 8 mm³, McDaniel et al., 2001), which leads to loss of signal intensity that extends the actual borders of the LV.

In the second labeling strategy we reduced the injected volume to 1.5 μl and the iron concentration to 0.67 mg Fe/ml (9.1 × 10⁵ particles). However, the final concentration of the contrast agent will be less than the injected solution, as the 1.5 μl fills less then 50% of the LV volume and the remaining CSF will dilute the contrast particles (volume of the LV is less than 8 mm³, McDaniel et al., 2001).

Results

Successful labeling of endogenous NPCs with MPIOs depends on the in situ labeling strategy

Intraventricular MPIO injection

Three weeks after intraventricular injection of 10 μl MPIOs (2.7 × 10⁵ particles; 3.00 mg Fe/ml), several hypointense (dark) spots could be detected along the RMS and in the center of the OB (Figs. 2A and B). This contrast relocation towards the OB was seen in all animals subjected to this injection method. Nevertheless, a prominent signal inhomogeneity was observed at the site of injection on the MR images (Figs. 2A and B). An injection of 10 μl completely fills the LV with iron oxide particles (volume of the LV is less than 8 mm³, McDaniel et al., 2001), which leads to loss of signal intensity that extends the actual borders of the LV.

In order to circumvent the dilution effect after intraventricular injection of MPIOs, we tested a labeling strategy where a small amount of MPIOs (9.1 × 10⁵ particles) was directly injected into the brain parenchyma just above the dorsal boundary of the RMS. Fig. 5A shows a sagittal in vivo MR image 3 weeks after MPIO injection. The 1.5 μl MPIO injection in the brain parenchyma is visible as a relatively large signal inhomogeneity and faint contrast spots are distinguishable along the RMS and in the OB. The location of the contrast seen on the in vivo MR image was confirmed by the corresponding ex vivo MR image (Fig. 5B). MPIO injection resulted in contrast relocation on the MR images in all mice, again with an accumulation of dark spots in the OB at later periods (number of hypointense spots: n = 17 at 3 weeks PI compared to n = 61 at 8 weeks PI).

Fig. 2. Sagittal 3D gradient-echo MR images of a mouse injected with 10 μl MPIOs in the LV (A and B) and a control mouse (C). Arrows indicate the routes of the RMS into the core of the OB. Hypointense spots are visible along the RMS and in the core of the OB on both the in vivo (A) and corresponding ex vivo (B) MR images of the same mouse 3 week PI. Notice the large susceptibility inhomogeneity located at the LV and surroundings in (A) and (B). The actual position of the LV is shown in (C).

Fig. 3. Sagittal 3D gradient-echo ex vivo MR images through the forebrain of mice 3 weeks after intraventricular injection with 1.5 μl MPIOs (A), 1.5 μl MPIOs-PLL (B) and 1.5 μl MPIOs-FUG (C). Hypointense spots along the RMS and in the core of the OB are only visible in (B). Notice the difference in susceptibility inhomogeneity in the LV visible in (B) compared to (A) and (C), although the same number of MPIOs (9.1 × 10⁵ particles) was injected.

Estimation of hypointense spots in the OB on the ex vivo MR images at the three measurement times (3, 5 and 8 weeks PI), showed that two out of the four intraventricular labeling strategies were successful (Fig. 4A). The number of dark spots was around zero for the 1.5 μl MPIOs and 1.5 μl MPIOs-FUG injections at 3 weeks PI but also at later PI times. Conversely, for the injections with either 10 μl MPIOs or 1.5 μl MPIOs-PLL, several dark spots were counted at 3 weeks PI and the number increased at later PI times. For these two strategies, the number of dark spots was at least nine times higher at 8 weeks than at 3 weeks PI. The minimum intensity projections (mIPs) at 8 weeks PI illustrate the difference between the two successful and the two non-successful strategies (Figs. 4B–E). On the mIPs of the 10 μl MPIO injection (Fig. 4B) and the 1.5 μl MPIOs-PLL injection (Fig. 4D), the route of the RMS is marked as a clearly discernible dark dotted line and numerous dark spots are present in the OB. This pattern of dark spots along the RMS and in the OB is not visible on the mIPs of the 1.5 μl MPIO injection (Fig. 4C) or the 1.5 μl MPIOs-FUG injection (Fig. 4E).

MPIO injection near the RMS
Fig. 4. (A) Estimation of the number of voxels representing hypointense spots in the core region of the ipsilateral OB at three different time points (3, 5 and 8 weeks) for four intraventricular labeling setups. Only for the 10 µl MPIOs and the 1.5 µl MPIOs-PLL injections, we could observe an accumulation of dark contrast spots in the OB over time. (B–E) The corresponding minimum intensity projections (mIPs) at 8 weeks PI, demonstrate the difference between the two successful and the two non-successful methods. On the mIPs of the 10 µl MPIO injection (B) and the 1.5 µl MPIOs-PLL injection (D) the RMS is delineated with dark spots and the OB clearly contains several contrast spots, while this is not the case for the 1.5 µl MPIO injection (C) or the 1.5 µl MPIOs-FUG injection (E).

Altogether, the MRI data showed that three in situ injection setups were successful for labeling endogenous NPCs with MPIOs: contrast relocation was seen in all mice subjected to the 10 µl MPIO injection in the LV, the 1.5 µl MPIOs-PLL injection in the LV and the 1.5 µl MPIO injection near the RMS (success rate of 100%, Table 3).

Intracellular uptake of MPIOs by endogenous NPCs

Immunohistochemistry and transmission electron microscopy were used to determine whether the MPIOs were incorporated in migrating NPCs. We performed double immunofluorescent stainings with antibodies for Dcx (migrating NPCs) and NeuN (mature neurons). Since the MPIOs contain a green fluorescent dye in their shell, they can be visualized on confocal laser scanning microscopic images. For the three MPIO labeling strategies where contrast relocation was seen on the MR images (10 µl MPIO injection in the LV, 1.5 µl MPIOs-PLL injection in the LV and 1.5 µl MPIO injection near the RMS), green fluorescent particles were observed in Dcx⁺ cells in the three parts of the SVZ-RMS-OB migratory pathway. In Figs. 6A–C are fluorescent images from mice injected with 10 µl MPIOs in the LV that show the presence of MPIOs at the level of the SVZ, RMS and OB. Proof of internalization of the contrast particles into the cell cytoplasm was provided by electron microscopy (Fig. 6D). These results are in agreement with previous immunohistochemistry analyses and electron microscopy described by Shapiro et al. (2006a) and Sumner et al. (2009).

Impact of in situ MPIO injection on adult neurogenesis

We examined the impact of intraventricular MPIO injection on cell proliferation in the SVZ at 1 day PI (effect of surgical injection and the presence of MPIOs in the LV) and 3 weeks PI (effect of the prolonged presence of MPIOs in the LV). The impact of MPIO injection near the RMS on NPC migration was investigated at 3 weeks PI. Furthermore, injection of MPIOs into the LV is likely to cause an inflammatory response, which can play an important role in the alteration of neurogenesis (Das and Basu, 2008). We evaluated the expression of immunocompetent cells bearing MHC-II in mice sacrificed 3 weeks after intraventricular MPIO injection and in control mice.

Intraventricular injection of MPIOs: 1 day PI

Statistical analysis of the quantification of BrdU-labeled cells showed significant differences between the three different groups in the SVZ ipsilateral to the injection (Kruskal–Wallis test: $\chi^2 = 8.23$, $p = 0.0167$ for mice injected with 10 µl MPIOs in the LV; $\chi^2 = 16.32$, $p = 1.36 \times 10^{-3}$ for mice injected with 1.5 µl MPIOs in the LV and 1.5 µl MPIO injection near the RMS; $\chi^2 = 6.1$, $p = 0.0137$ for mice injected with 1.5 µl MPIOs-PLL injection in the LV). The number of BrdU-labeled cells was significantly reduced after MPIO injection compared to the control mice as shown by the immunohistochemistry analyses and electron microscopy described by Shapiro et al. (2006a) and Sumner et al. (2009).

Table 3

Overview of the five different in situ labeling strategies with number of mice and success rate (% of number of mice where contrast relocation was visualized by in vivo and ex vivo MRI).

<table>
<thead>
<tr>
<th>MPIOs</th>
<th>10 µl LV</th>
<th>1.5 µl LV</th>
<th>1.5 µl MPIOs-PLL</th>
<th>1.5 µl MPIOs-FUG</th>
<th>1.5 µl RMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPDIs</td>
<td>$2.7 \times 10^7$ part.</td>
<td>$9.1 \times 10^7$ part.</td>
<td>$9.1 \times 10^7$ part.</td>
<td>$9.1 \times 10^7$ part.</td>
<td>$9.1 \times 10^7$ part.</td>
</tr>
<tr>
<td>MPDIs-PLL</td>
<td>$1.7 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
</tr>
<tr>
<td>MPDIs-FUG</td>
<td>$1.7 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
<td>$4.1 \times 10^7$ part.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>n = 4 + 3</th>
<th>n = 4 + 2</th>
<th>n = 4 + 0</th>
<th>n = 4</th>
<th>n = 4 + 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Success rate</td>
<td>100%</td>
<td>17%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
P = 0.016; Fig. 7). Post hoc tests revealed a significant decrease in the number of BrdU+ cells in mice injected with MPIOs compared to the control group (Mann–Whitney test: Z = -2.12, P = 0.034) and the sham group (PBS injection, Mann–Whitney test: Z = -2.02, P = 0.043). In addition, the sham group showed a decreased number of BrdU-positive cells compared to the control group (Mann–Whitney test: Z = -2.12, P = 0.034). Also the contralateral SVZ revealed differences between the three groups (Kruskal–Wallis test: χ² = 7.12, P = 0.027; Fig. 7). A decreased number of BrdU-labeled cells in the MPIO-injected mice was found compared to the other groups (versus the control group, Mann–Whitney test: Z = 2.12, P = 0.034; versus the sham group, Mann–Whitney test: Z = -2.31, P = 0.021).

Intraventricular or RMS injection of MPIOs: 3 weeks PI
Remarkably, in mice injected with 10 μl MPIOs in the LV, large clusters of MPIOs were visible in the LV ipsilateral to the injection. In these mice (n = 4), BrdU+ cell counts around this ventricle were not feasible as the MPIO clusters masked the BrdU-labeled nuclei (Fig. 8B). For the remaining three groups, statistical analysis of BrdU+ cell quantification showed significant differences between the groups in the SVZ ipsilateral to the injection site (Kruskal–Wallis test: χ² = 7.06, P = 0.029; Fig. 8C). We observed a decreased number of BrdU+ cells in the MPIOs-PLL-injected mice compared to the MPIO-injected mice (Mann–Whitney test: Z = -2.12 and P = 0.034). This result was confirmed at the contralateral SVZ (Mann–Whitney test: Z = -2.12, P = 0.034).

Statistical analysis of the quantification of BrdU-labeled cells in the OB at 3 weeks after RMS injection showed that the density of BrdU+ cells in the OB ipsilateral to the RMS injection was significantly lower in the MPIO-injected mice compared to the sham group (Mann–Whitney test: Z = -2.24, P = 0.025, Fig. 9).

Immunohistochemistry and electron microscopy from a mouse injected with 10 μl MPIOs in the LV. A–C Fluorescence images show the presence of green fluorescent particles (MPIOs) in the SVZ, the RMS and the OB. Dcx+ (red staining) and NeuN+ (blue staining) cell containing MPIOs (white arrow) in the OB is shown in (C), the double staining indicates that this NPC is differentiating into a mature neuron. D Electron microscopic image at the level of the SVZ shows iron containing vesicles (black arrow) located in the cytoplasm near the nucleus of an astrocyte-like progenitor cell. LV, lateral ventricle.

**Fig. 6.** Immunohistochemistry and electron microscopy from a mouse injected with 10 μl MPIOs in the LV. (A–C) Fluorescence images show the presence of green fluorescent particles (MPIOs) in the SVZ, the RMS and the OB. Dcx+ (red staining) and NeuN+ (blue staining) cell containing MPIOs (white arrow) in the OB is shown in (C), the double staining indicates that this NPC is differentiating into a mature neuron. (D) Electron microscopic image at the level of the SVZ shows iron containing vesicles (black arrow) located in the cytoplasm near the nucleus of an astrocyte-like progenitor cell. LV, lateral ventricle.

**Fig. 7.** Number of BrdU-positive cells in the SVZ 1 day after intraventricular injection. MPIO-injected animals showed significantly less BrdU+ cells than sham-operated and control mice in the ipsilateral and contralateral SVZ. Sham-operated mice showed significantly less BrdU+ cells than control mice only in the ipsilateral SVZ. Error bars represent the standard error of the mean and the asterisks indicate a significant difference (*P < 0.05).

**Fig. 8.** BrdU histology and quantification in the SVZ 3 weeks after intraventricular injection. Light microscopic coronal images at the level of bregma of mice 3 weeks after injection of 1.5 μl MPIOs (A) or 10 μl MPIOs (B) in the left LV. BrdU+ cells are stained brown and are visible along the ventricular wall in (A). The presence of MPIO clusters masks the BrdU-labeled cells in the left ventricular wall in (B). (C) Number of BrdU-positive cells in the SVZ 3 weeks after intraventricular injection. MPIOs-PLL-injected mice showed significant less BrdU+ cells than MPIO-injected mice in the SVZ ipsilateral and contralateral to the injection. Error bars represent the standard error of the mean and the asterisks indicate a significant difference (*P < 0.05). LV, lateral ventricle; CC, corpus callosum.
animals, only a few MHC-II-stained cells were faintly visible in the choroid plexus (Figs. 10 K, L), while in mice injected with 1.5 μl MPIOs-PLL or 1.5 μl MPIOs, several distinct MHC-II-stained cells were seen (Figs. 10 E, F and H, I). Even more MHC-II-positive cells were found in the animals injected with 10 μl MPIOs (Figs. 10 B, C). Notably, MHC-II-positive cells were found to be in close contact with the particles. No MHC-II-positive cells were detected at the anterior horn of the LV in any of the animals (data not shown).

Discussion

Labeling endogenous NPCs in the adult rodent brain with MR contrast agents offers a unique method to investigate neural recruitment with MRI. An important application lies in the in vivo monitoring of deviating NPC migration patterns and new cell therapies for several neurodegenerative diseases, in which the available animal models are mainly (transgenic and knockout) mouse models. Thus far in situ labeling of endogenous NPCs with MR contrast agents was only accomplished in rats (Panizzo et al., 2009; Shapiro et al., 2006a; Sumner et al., 2009). This paper demonstrates that in situ labeling of endogenous NPCs with MPIOs and visualization of NPC migration with in vivo MRI are also possible in the adult mouse brain. Several in situ labeling strategies are described in this paper. Endogenous NPC labeling was initially achieved by injection of a large number of MPIOs (2.7 × 10^7 particles) into the LV near the SVZ. The major drawback of this labeling method remains the large signal inhomogeneity which hampers the visualization of the beginning of the RMS on the MR images (see Fig. 2). In an attempt to reduce this artifact, new in situ labeling strategies using a smaller amount of particles (9.1 × 10^5 particles) were tested. We succeeded in preserving endogenous NPC labeling and, moreover, visualizing the entire route of the RMS, when a small number of

![Fig. 9. Number of BrdU-positive cells in the OB ipsilateral and contralateral to the RMS injection. MPIO-injected animals showed significantly less BrdU+ cells/mm^3 than sham-operated mice in the OB ipsilateral to the injection. Error bars represent the standard error of the mean and the asterisks indicate a significant difference (*P<0.05).](image)

![Fig. 10. Histological images for the immunodetection of MHC-II-presenting cells. Each column represents slices at different locations along the ventricular system: column 1 at the level of bregma; column 2 at the level of the third ventricle and column 3 at the level of the hippocampus. Each row represents a different intraventricular labeling strategy: (A–C) 10 μl MPIO injection; (D–F) 1.5 μl MPIOs-PLL injection; (G–I) 1.5 μl MPIOs injection; (J–L) no injection. In the first column, MHC-II-stained cells are only visible in (A) and (D). In the other two columns, many MHC-II-stained cells are present in (B, C). Fewer MHC-II-positive cells are visible in (E, F) and (H, I), while just a faint staining is visible in (K) and (L). Insets are the magnifications of the delimited areas. White scale bars are 500 μm; all black scale bars are 100 μm.](image)
particles combined with the transfection agent PLL was injected into
the LV. In agreement with the results of Panizzo et al. (2009), our MRI
data suggest that addition of a cationic protein-based transfection
agent contributes to a more successful labeling. It is believed that the
ventricular wall possess a significant affinity for positively charged
contrast agents (Wan et al., 1992). The electrostatic interaction
between the positively charged PLL and the large negatively charged
domains of the ventricular wall ensures a better cellular uptake of the
contrast particles. The observation of a larger signal inhomogeneity in
the LV after the MPIOs-PLL injection (see Fig. 3) presumably reflects a
better distribution of the MPIOs-PLL complexes through the LV as a
result of the previous mentioned electrostatic forces. The contradic-
tory result of the MPIOs-FUG complex suggests that this multi-
component reagent did not improve in vivo transfection. Labeling of
endogenous NPCs was also achieved through injection of a small
number of particles outside the LV, i.e., directly into the brain
parenchyma touching the dorsal boundary of the RMS. Two factors
may have contributed to the improved labeling after RMS injection
compared to the injection of a small number of particles in the LV.
First, the dilution effect of MPIOs in the CSF that occurs after
intraventricular injection is eluded. Besides that, MPIOs are in direct
contact with endogenous NPC after RMS injection, whereas for the
intraventricular injection the ependymal cell layer lining the
ventricular wall functions as a barrier between the MPIOs and the
NPCs in the SVZ. Nevertheless, due to the injection coordinates of this
approach, the RMS was partially covered by the induced area of signal
loss on the MR images.

It is known that almost 30,000 newly generated cells daily enter
the RMS from the SVZ (Alvarez-Buylla et al., 2001). Furthermore, SVZ-
derived NPCs may reach the granular and the periglomerular layer of
the OB within one week of migration (Lois and Alvarez-Buylla, 1994;
Petreanu and Alvarez-Buylla, 2002; Saghatelany et al. 2004; Suzuki
and Goldman, 2003). A previous study on long-term in vivo marking
of SVZ stem cells by lentiviral vectors has demonstrated that marked
SVZ-derived migrating progenitor cells accumulated in the OB over
time up to 7 months (Geraerts et al., 2006). Even though our
longitudinal MRI data and contrast spot estimations demonstrate the
accumulation of dark spots into the OB over time, at 3 weeks PI, only
a few contrast spots were visible in the OB. It has been previously
demonstrated that an isotropic resolution of 100 µm should be
sufficient to detect single cells labeled with MPIOs in the rodent brain
(Shapiro et al., 2005). Even single MPIOs detection at the same
resolution has already been reported (Shapiro et al., 2004). In a
previous report, a similar delay in accumulation of MPIO-labeled cells
in the OB was observed (Shapiro et al., 2006a). The authors suggested
that this could be due to a slow uptake of the particles from the
ventricle. Nevertheless, this delay could also indicate a hampered
genesis as a result of in situ cell labeling.

Many studies have used MR contrast agents to explore in vitro the
effect of cell labeling on cellular functions, such as viability, proliferation
and differentiation capacity (Arbab et al., 2003; Frank et al., 2003;
Jendelova et al., 2003). However, little is known about the effects exerted
by MR contrast agents on cellular functions after in situ cell labeling. In
the adult brain, stem cells reside in a specialized microenvironment, also
called ‘neurogenic niche’. An alteration in this microenvironment leads
to alteration in the process of neurogenesis. Neuroinflammation can
influence adult neurogenesis (Das and Basu, 2008). Infiltration of
inflammatory cells proceeding from the choroidplexus has been
reported after intraventricular injection of neurominidase (Del Carmen
Gomez-Roldan et al., 2008) or a high dose of rhodamine microspheres
(Carbonell et al., 2005). Intraventricular injection of MPIOs is likely
to induce a similar inflammatory response. Our study finds evidence for an
inflammatory reaction at 3 weeks after intraventricular MPIO injection.
MHC-II-positive cells were clearly present in the choroid plexus of the
ventricular system of mice injected with MPIOs. In the reports of Del
Carmen Gomez-Roldan et al. (2008) and Carbonell et al. (2005),
ependymal damage preceded the infiltration of inflammatory cells and
the effect on the ependyma was thought to be dose-dependent. In
addition, in the study of Carbonell et al. (2005), it is suggested that the
selective uptake of microspheres by the ependymal cells was responsi-
ble for ependymal death. We also observed a dose-dependent effect.
The highest amount of MHC-II-positive cells was observed after
intraventricular injection of a large number of MPIOs. A limited number
of MHC-II-positive cells were observed after intraventricular injection of
a small amount of MPIOs. Addition of PLL to the MPIOs slightly increased
the inflammatory response. As mentioned previously, PLL addition
contributed to a more successful labeling due to improved cellular
uptake of the MPIOs. This data support the assumption that a higher
particle uptake will lead to increased ependymal death and as a result
increases the inflammatory response. In fact, the degree of particle
uptake and subsequently ependymal death may have an important role
in the success rate of the MPIO labeling strategy. After ependymal
damage and detachment, cells of the SVZ are exposed to the content of
the lateral ventricle, including the injected particles. This may lead to
improved labeling of the NPCs in the SVZ with MPIOs. Proinflammatory
cytokines produced by the ependymal cells and intraventricular cells
have been suggested to be responsible for ventricular inflammation (Del
Carmen Gomez-Roldan et al., 2008). Furthermore, many proinflam-
matory cytokines are known to be negative regulators of NPC prolifera-
tion (for a review see Das and Basu, 2008) and thus, it is not surprising that
our BrdU quantification showed a declined proliferation in the SVZ 1 day
after intraventricular injection of even a small number of MPIOs. At 3
weeks PI, our BrdU results show that the proliferation in the SVZ has
recovered, even slightly increased, after injection of a small number of
MPIOs. This result supports the hypothesis that, after ependymal
damage, proliferation in the SVZ can be triggered by the presence of
proliferation-stimulating factors in the CSF (Del Carmen Gomez-Roldan
et al., 2008). While the BrdU data at 3 weeks did not showed a significant
difference in cell proliferation between the MPIOs-PLL and control
animals, our data suggest a negative effect on proliferation in the SVZ
when the transfection agent PLL is added to MPIOs. We also observed
impeded NPC migration after injection of MPIOs near the RMS, which
seemed to be caused by the presence of MPIOs and not by the surgical
procedure. It has been previously suggested by Modo et al., (2005) that
MPIOs, while inert inside a cell because of their styrene/divinylbenzene
coating, could have significant impact on cellular processes due to their
large particle size.

Since intraventricular labeling strategies probably exert a detri-
mental effect on ependymal cells, the time delay in accumulation of
dark spots into the OB could also be explained by disturbed CSF
movement. It has indeed been demonstrated that migration of NPCs is
guided by movement of the CSF in the LVs, caused by the beating of
ependymal cilia (Sawamoto et al., 2006). In addition, changes in the
viscosity of the CSF have been reported to decrease the ciliary beat
frequency (O’Callaghan et al., 2008). It is thus possible that the
presence of MPIOs in the CSF alters the CSF movement and consequently
alters the NPC migration.

In conclusion, labeling of endogenous NPCs in the adult mouse
brain by in situ injection of MPIOs into the LV or near the RMS is
feasible and the migration of labeled NPC can be visualized with in
vivo MRI. From the in situ labeling strategies presented in this report,
intraventricular injection of a small number of particles combined
with the transfection agent PLL turned out the best method because
labeling of the NPCs was successful and the entire migration route was
visualized with MRI, while proliferation in the SVZ was only
marginally affected. Future studies should explore the efficiency of
this in situ labeling method to visualize aberrant NPC migration in
various mouse models for brain injury and neurodegeneration with in
vivo MRI. Furthermore, the findings in the current study indicate that
future studies addressing the development of new in situ labeling
strategies should include detailed analysis of the possible impact of
the labeling strategies on adult neurogenesis.
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