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Research article

Neural stem cells from mouse strain Thy1 YFP-16 are a valuable tool to monitor and evaluate neuronal differentiation and morphology

Ivan Alić[a,b], Nina Kosi[a], Katarina Kapuralin[c], Dunja Gorup[a], Srećko Gajović[a], Roland Pochet[d], Dinko Mitrečić[a,*]

[a] Croatian Institute for Brain Research, School of Medicine, University of Zagreb, 10 000 Zagreb, Croatia
[b] Faculty of Veterinary Medicine, University of Zagreb, 10 000 Zagreb, Croatia
[c] New York University Abu Dhabi, United Arab Emirates
[d] Université Libre de Bruxelles, 1070 Bruxelles, Belgium

HIGHLIGHTS

- Thy1-YFP+ cells allow follow up of neuronal birth and fates during embryo development.
- Thy1-YFP+ neural stem cells allow *in vitro* analyses of cells' features and potential.
- Thy1-YFP+ neural stem cells are a robust tool to follow cells after transplantation.

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ABSTRACT

To analyse events following transplantation of stem cells in the brain robust tools for tracing stem cells are required. Here we took advantage of the mouse strain B6.Cg-Tg(Thy1-YFP)16Jrs/J (Thy1 YFP-16), where yellow fluorescent protein (YFP) is under control of the promoter of *Thy1* gene. This allows visualising whole neurons, *i.e.* their cell body, axons and dendrites. In this work fluorescent cells were followed during embryonic development, *in vitro* differentiation, and after transplantation in the healthy and stroke-affected mouse brain. During embryonic development Thy1-YFP positive cells were first observed on E12.5 and subsequently located in the prosencephalon, rhombencephalon, spinal cord and peripheral nerves. Quantitative analysis by RT-PCR and immunocytochemistry revealed that Thy1-YFP positive cells during embryo development and *in vitro* differentiation were expressing nestin and SOX2 then MAP2, β3-tubulin and NeuN. Thy1-YFP positive cells isolated from E14.5 represented 21.88 ± 053% (SD) of the cultivated neurons and this remained constant along *in vitro* differentiation. On the other hand, proportion of Thy1-YFP positive cells reached 50% of neurons in perinatal and one month old mouse brain. Neural stem cells isolated from Thy1 YFP-16 mouse strain transplanted near hippocampus of the healthy and stroke-affected brain were distinguishable by YFP fluorescence. They differentiated into mature neurons and were detectable even 14 weeks after transplantation, the end point of our experiment. In conclusion, stem cells originating from Thy1 YFP-16 mice represent an outstanding tool to monitor neurogenesis enabling morphological analyses of new neurons and their projections, in particular after transplantation in the brain.

1. Introduction

Beneficial effects of stem cells and stem cell-derived neurons and glia after transplantation in the nervous tissue provide a strong rationale for cell therapy strategies for brain repair. To get insight in events following stem cells transplantation, including their migration, survival and differentiation, robust tools to trace stem cells are required. Commercially available exogenous dyes fade after several weeks and the signal dilutes [1,2]. On the other hand, majority of mouse lines characterized by presence of fluorescent proteins in neurons do not allow visualization of all cell projections and the

* Corresponding author at: Laboratory for Stem Cells, Croatian Institute for Brain Research, School of Medicine, University of Zagreb, 10 000 Zagreb, Croatia.
E-mail addresses: ialic@vef.hr (I. Alić), ninakosi@hiim.hr (N. Kosi), katarina.kapuralin@nyu.edu (K. Kapuralin), dunja.gorup@hiim.hr (D. Gorup), srecko.gajovic@hiim.hr (S. Gajović), rpochet@ulb.ac.be (R. Pochet), dinko.mitrecic@mef.hr (D. Mitrečić).

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signal present in cells is not linked to the stage of their differentiation [3]. Design of mouse expressing fluorescent reporter, under control of neuron specific *Thy1* gene promoter, offered the possibility to specifically trace all parts of neurons, including the cell body, axon and dendrites [4]. Thy1 is a highly conserved cell surface protein belonging to immunoglobulin superfamily. It was firstly discovered in thymus, where it marks precursors of T lymphocytes (THYmocyte differentiation antigen 1) [5]. In addition, it has been discovered in fibroblasts, endothelial cells, smooth muscles and some subsets of neurons. Based on its involvement in cell–cell and cell-matrix interactions, it is linked to cell and apoptotic signalling, leukocyte adhesion, tumor suppression and fibroblast proliferation [4–7]. Nevertheless since in B6.Cg-Tg(Thy1-YFP)16Jrs/J (Thy1 YFP-16) yellow fluorescent protein is expressed at high levels in a subset of neurons [4,6,7] we hypothesized that fluorescence depicting the whole neuron can be used as a powerful tool to analyse differentiation of neural stem cells, in particular after transplantation. Presented study took systematic approach to achieve quantitative description of the fluorescent cells obtained from Thy1 YFP-16 mice during neuronal differentiation both *in vivo* and *in vitro*. In addition, this study set a goal to compare cell fates in cell culture to events after the transplantation of cells into the brain of experimental animals, as well as with the events that occur during normal embryonic development. Moreover, we could trace neural stem cells transplanted in the healthy and stroke-affected mouse brain. The analysis of birth and fates of neurons during embryonic development, *in vitro* differentiation and after transplantation in the healthy and stroke-affected brain indicated that Thy1-YFP cells can be used as a robust tool to monitor neural stem cells and to evaluate their differentiation by visualizing their growing projections.

2. Materials and methods

2.1. Animals and housing

Two mice strains have been used: B6.Cg-Tg(Thy1-YFP)16Jrs/J (Thy1 YFP-16) and C57Bl/6NCrl (wild type control) (The Jackson Laboratory, Bar Harbor, ME, USA), kept on the same background. The animals were kept at the animal facility at the Croatian Institute for Brain Research at the temperature $22 \pm 2\,^{\circ}C$, with $55\% \pm 10\%$ humidity, and 12/12 h light/dark cycle. Water and pelleted food were given *ad libitum*. All experiments on animals described in this work received approval of the Internal Review Board of the Ethical Committee of the School of Medicine University of Zagreb (04-77/2010-238) and Faculty of Veterinary Medicine (251/61-01/139-13-4) and they were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Neural stem cells and cell culture

For isolation of cells pregnant Thy1 YFP-16 females were sacrificed and neural stem cells were isolated from telencephalic wall of E14.5 fetuses using Accutase (Gibco by Life Technologies, A11105-01). Isolated cells were placed into flasks (BD Falcon, 353110) in a specific proliferation supporting medium comprising of: DMEM/F-12 (Gibco by Life Technologies, 31331-028), B-27 Supplement (Gibco by Life Technologies, 17504-044), N-2 Supplement (Gibco by Life Technologies, 17502-048), Penicillin Streptomycin (Gibco by Life Technologies, 15070-063), FGFb (Recombinant Mouse Fibroblast Growth Factor-basic, PMG0035) and EGF (Recombinant Mouse Epidermal Growth Factor, PMG8041). Cells were cultivated in suspension, and after two days neurospheres were formed. For the purpose of transplantation, neurospheres were dissociated by Accutase and 1 million of cells were transplanted in 1 μL of artificial CSF. For the purpose of differentiation analyses neurospheres were dissociated, single cells and small neurospheres were plated on 12 mm coverslips (200–250 000 cells per coverslip). Coverslips were previously coated with Poly-D-lysine (500 μg/mL, 24 h at 37 °C, SIGMA, P6407-5MG) and laminin (10 μg/mL, 24 h at 37 °C, SIGMA, L2020-1MG). Cells used for *in vitro* differentiation were plated in a medium without proliferation factors. After 24 h the medium was changed into: Neurobasal (Gibco by Life Technologies, 21103-049) which supports neurons *in vitro* and antibiotic with glutamine (Pen Strep Glutamine, Gibco by Life Technologies, 10370-016). Cells were fixed with 4% PFA (pH 7.4) on the 1st, 3rd, 5th and 7th day of differentiation.

2.3. RT-PCR

Total RNA was isolated by using commercial RNeasy® Mini Kit (Qiagen) following manufacturer instructions. After RNA isolation, concentration was calculated on spectrophotometer (Nanodrop, Thermofisher Scientific) and by using high capacity RNA-to-cDNA Kit (Applied Biosystems), RNA was converted in cDNA. RT-PCR was performed on cells in the same time points for three assays: nestin (Mm00450205_m1), MAP2 (Mm00485231_m1) and GFAP (Mm01253033_m1). As housekeeping gene, we used β-Actin (ACTB MGB 4352933E) (TaqMan Gene Expression Assays). All samples were made in triplicate with 1 μg cDNA in total volume 20 μL using Applied Biosystems 7500 Real-Time PCR System. Relative quantification was made using formula $2^{-\Delta CT}$.

2.4. Mouse stroke model by middle cerebral artery occlusion (MCAO)

Ischaemic brain injury was induced by transient left middle cerebral artery occlusion (MCAO) in wild type mice, age 3 months and weight 25–30 g. Operation was performed in inhalation anaesthesia, mixture 2% isoflurane in 100% O_2. Surgery was performed under dissection microscope (Zeiss, Stemi DV4 Spot). During surgery animal body temperature was maintained with heating pad. Following ventral neck surgery and blood vessels preparation, intraluminal filament (Doccol Company) was inserted through common carotid artery (CCA) into internal carotid artery (ICA) to the origin middle cerebral artery (MCA) and left 90 min. After 90 min intraluminal filament was withdrawn, perfusion was restored and animal intraperitoneally received analgetic buprenorphine (0.03 mg/kg).

2.5. Stereotaxic transplantation of neural stem cells into the mouse brain

Cells for transplantation into mouse brain were labelled with PKH26 fluorescent dye (PKH26GL-1KT, PKH26 Red Fluorescent Cell Linker Kit for general cell membrane labelling, SIGMA, 081M0964) following manufacturer's instructions.

For stereotaxic transplantation we used two groups of mice: healthy and stroke-affected. In both groups animals were 3 months old and weighted 25–30 g. Stereotaxic coordinates (AP −1.3, ML +2.0 and DV −1.5) were determined according to stereotaxic atlas [8] and injections were performed using KOPF stereotaxic apparatus (900LS) and Hamilton syringe needle 1 μL. For anaesthesia, Avertin (Sigma, T48402-5G) was used at a dose of 0.5 g/kg, given intraperitoneally. After transplantation animals were kept on the heating pad and then they were returned to their cage.

2.6. Animal sacrifice, perfusion, fixation and tissue isolation

Wild type mice were anesthetized using Avertin (0.5 g/kg) and then perfused transcardially with PBS and subsequently 4%

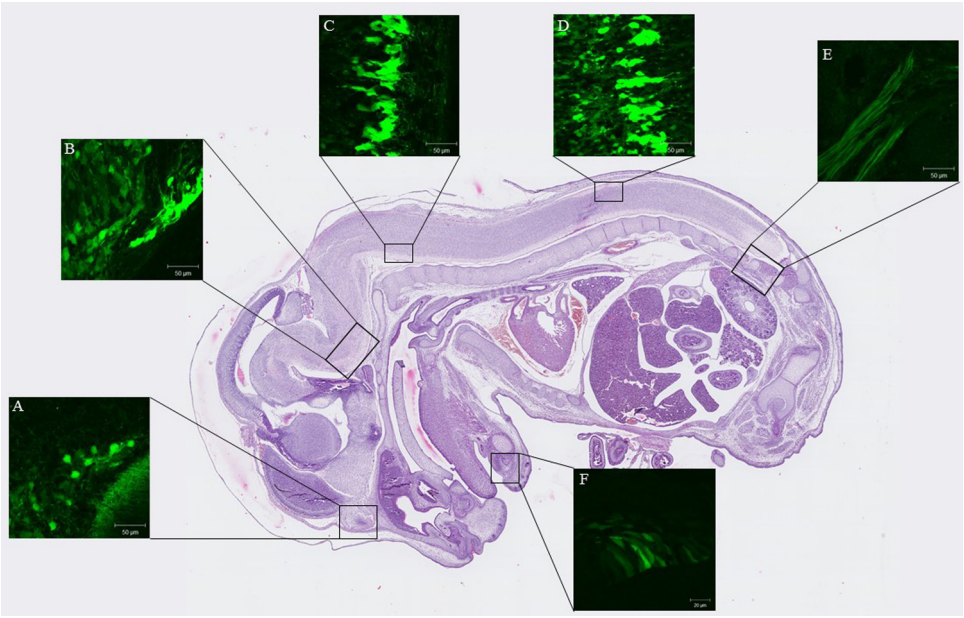

Fig 1. Thy1-YFP expression at E14.5. Positive cells were observed in developing olfactory bulb (A), ponotomedullary part of rhombencephalon (B), ventral (C) and dorsal (D) horn of the spinal cord. In peripheral nervous system positive cells were observed in developing spinal nerves (E) and in the cap stage of tooth (F).

paraformaldehyde in PBS (pH 7.4). Brains were isolated and further fixed by immersion in the same fixative at 4 °C overnight. They were washed in PBS and transferred to 10% sucrose followed by 30% sucrose in PBS at 4 °C. Frontal 20 μm thick sections were cut with a cryostat and mounted on Superfrost Plus (Menzel Glaser) coated slides.

Pregnant Thy1 YFP-16 females, at different stages of gestation (from 9th to 17th day) were used for embryo isolation. After sacrifice of pregnant animal, abdominal cavity was opened and the embryos were isolated by opening uterine wall and foetal membranes. Age of isolated embryos was confirmed by checking outer features specific for each stage, then fixed in 4% PFA (pH 7.4) and cryosectioned on 20 μm thick slices as described above.

2.7. Immunofluorescence

Cells and tissues were immunolabelled with specific primary antibodies as followed: as stem cell markers – nestin (mouse monoclonal ab, diluted 1:200, Millipore, MAB353) and SOX2 (rabbit polyclonal ab, diluted 1:200, Novus Biologicals, NB110-37235); for neurons MAP2 (chicken polyclonal ab, diluted 1:1000, Abcam, ab5392), β3-tubulin (rabbit monoclonal ab, diluted 1:200, Cell Signaling, D71G9) and NeuN (mouse monoclonal ab, diluted 1:200, Millipore, MAB377) and for astrocytes GFAP (chicken polyclonal ab, diluted 1:250, Abcam, ab4674). Primary antibodies were diluted in 0.2% Triton X-100 (Sigma. T8787-100ML) in PBS and 3% goat serum at 4 °C overnight. Next day primary antibodies were rinsed in PBS and samples were further immunolabelled with fluorescent secondary antibodies as followed: Alexa Fluor 546 goat anti-mouse (Invitrogen, A11003), Alexa Fluor 546 goat anti-rabbit (Invitrogen, A11010), Alexa Fluor 546 goat anti-chicken (Invitrogen, A11040) and Alexa Fluor 633 goat anti-rabbit (Invitrogen, A21070). Secondary antibodies were diluted 1:1000, except Alexa Fluor 633 goat anti-rabbit (1:500) in 0.2% Triton X-100 in PBS. Secondary antibodies were rinsed with PBS and DAPI (Roche) was used as nuclear counterstain. Finally, cells and tissues were rinsed in PBS and coverslipped with Dako Fluorescent Mounting Medium (S3023). Fluorescent analysis was made with confocal microscope Zeiss LSM 510 Meta.

2.8. Cell counting and quantification

Number of positive cells was counted on photographs obtained by confocal microscope (Zeiss LSM 510 Meta) in ten fields per coverslip in three independent experiments. For quantification of cells *in vitro*, days 0, 1, 3, 5 and 7 were analysed. Since Shapiro-Wilk test revealed normal distribution of samples at different time-points and ANOVA found no statistically significant differences between the time-point groups, we calculated average mean ± standard deviation of both MAP2 and Thy1. As a limit of statistical significance P value < 0,01 was accepted. During embryonic development we analysed stages from E12.5 to P0, neonates, 7 days and one month old mice. Embryonic structures were determined and compared with online atlas [9]. After we defined E17.5 as the referral point, we counted cells in all regions of that embryo stage: "–" refers to no signal, "+/–" to signal present in less than 10% of cells, "+" to signal present in 10–20% of cells, "++" to signal present in 20–35% of cells, "+++" to signal present in 35–50% of cells. Other stages were compared to the referral point and evaluated semi-quantitatively. The results were rated independently by two evaluators and coincided 100%.

3. Results

3.1. Thy1-YFP cells allow follow up of birth and differentiation of neurons during embryonic and early postnatal development

To analyse expression of Thy1 during embryonic and early postnatal development we have analysed stages from E12.5 (onset of Thy1 expression) to E17.5, then in neonates, 7 days and in one month old mice (Fig. 1, Table 1). The first Thy1-YFP positive cells were observed on E12.5 in the central and peripheral nervous system. In the central nervous system Thy1-YFP positive cells were observed in the developing spinal cord, mostly in the future ventral horns and some scattered cells in the developing brain (Fig. 2A–C).

Table 1
Thy1-YFP expression during mice embryonic development. E17.5 has been defined as the referral point and at that stage number of cells have been counted: "–" refers to no signal, "+/–" to signal present in less than 10% of cells, "+" to signal present in 10–20% of cells, "++" to signal present in 20–35% of cells, "+++" to signal present in 35–50% of cells. Other stages were compared to the referral point and evaluated semiquantitatively. "PNS" peripheral nervous system, "E" embryonic day, "P" newborn, "W1" one week old pups, "M1" one month old mouse.

Neural system	E12.5	E13.5	E14.5	E15.5	E16.5	E17.5	P0	W1	M1
CNS									
Brain									
Prosencephalon	+/–	+/–	+	+	++	++	++	+++	+++
Mesencephalon									
Rhombencephalon	+	+	++	++	+++	+++	+++	+++	+++
Spinal cord									
Ventral horn	+	++	++	+++	+++	+++	+++	+++	+++
Dorsal horn	+/–	+	+	++	+++	+++	+++	+++	+++
PNS									
Cranial nerves			+	++	++	++	++	+++	+++
Spinal nerves	+	+	++	++	++	++	++	+++	+++
Retina						+	++	+++	+++

On E13.5 number of Thy1-YFP positive cells increased in the ventral horns, and to smaller extent in the dorsal horns of the spinal cord. In the brain, positive cells were observed in the pontomedullary part of rhombencephalon and some cells in olfactory bulbs (Table 1). During E14.5 number of Thy1-YFP positive cells was higher than a day before (Fig. 2D–I, Table 1). Apart from already mentioned structures, positive cells were observed in the cranial and spinal nerves and in the developing tooth (Fig. 1). On E15.5 and E16.5 positive cells exhibited morphology of mature neurons and the signal present in cell extensions, including dendritic spines (Fig. 2J–L). On E17.5, apart from described structures, Thy1-YFP positive cells were observed in retina. Also, on E17.5 Thy1-YFP positive cells were observed in CA1 region of hippocampus (Table 1).

To analyse types of cells detected by Thy1-YFP during embryo development, immunohistochemical staining was performed on E12.5, E14.5 and E16.5. While *in vitro* co-localization of nestin and Thy1-YFP was detected (see below), we did not observe such co-localization in *in vivo*. In general, nestin positive cells were Thy1-YFP negative and they were mostly visible on E12.5 (Fig. 2A–C) where they had a spindle-shaped morphology. With nervous system maturation, number of nestin positive cells gradually decreased. SOX2 positive cells were present in all three examined stages and they co-localized with Thy1-YFP (Fig. 2G–I). MAP2 (Fig. 2D–F and J–L) as well as β3-tubulin (not shown) were also positive and revealed co-localization with Thy1-YFP positive cells.

In perinatal mouse (P0) THY1-YFP positive cells were observed in the brain cortex and subventricular zone.

In one week old mouse brain Thy1-YFP positive cells were present in olfactory bulb (Fig. 3A), in the CA1 region of hippocampus (Fig. 3B) and in the brain cortex (Fig. 3C).

In one month old mouse, brain was completely developed and cells extended a large number of their projections. Cells in olfactory bulb were more branched than in one week old mouse (Fig. 3D), in the hippocampus positive cells were present in CA1 and CA3 region (Fig. 3E). In the cortex, 50% of Map2 and NeuN positive neurons were Thy1-YFP positive (Fig. 3F). Furthermore, Thy1-YFP positive cells were present in thalamic nuclei. In the peripheral nervous system, positive cells were present in retina and in all cranial and spinal nerves including nerves of brachial and sacroiliac plexus.

3.2. *Thy1-YFP cells allow follow up and detailed morphological analyses of differentiation of neural stem cells* in vitro

To describe differentiation of Thy1-YFP positive cells and appearance of the signal *in vitro* we used neural stem cells cultures obtained from YFP-16. We analysed the progress of differentiation on both RT-PCR and immunocytochemical level. Already during the first day of differentiation Thy1-YFP positive cells were present in a form of rounded progenitor cells with lower fluorescent signal and in a form of more mature cells with a bright fluorescent signal (Fig. 4A–C). RT-PCR analyses revealed that on day 0 expression of nestin was the highest and there was as well signal of MAP2 present (Fig. 5C). Immunocytochemical analyses confirmed that scarce more mature cells were SOX2, MAP2, β3-tubulin and NeuN positive but they were not nestin positive. Thy1-YFP positive progenitor cells exhibited co-localization with nestin. Both Thy1-YFP positive progenitor and mature neurons were SOX2 positive (not shown). The percentage of cells co-expressing Thy1-YFP and neuronal markers (MAP2, β3-tubulin) during the first day of differentiation was 22.5 ± 0.4% (Fig. 5A).

On the third day of differentiation RT-PCR analyses revealed that expression of nestin dropped for 40% in comparison to day 0 and that expression of MAP2 doubled (Fig. 5B,C). Indeed, cell morphology suggested progression toward more differentiated cell types. Number of nestin positive cells in culture decreased from 99% to 30% while proportion of neuronal cells increased to 70% when compared to the previous stage (Fig. 5B,C). On this stage we detected some GFAP-expressing cells, although immunocytochemistry was still not able to visualize them. The percentage of cells co-expressing Thy1-YFP and neuronal markers (MAP2, β3-tubulin) on the third day of differentiation was 21.3 ± 0.5% (Fig. 5A).

On the fifth day of differentiation the decrease of expression of nestin and increase of MAP2 continued. Cells positive for neuronal markers appeared as mature and highly branched. The percentage of cells co-expressing Thy1-YFP and neuronal markers (MAP2, β3-tubulin) was 21.9 ± 0.5% (Fig. 5A). GFAP positive cells were observed on day 5 as 1% of a total number cells/coverslip, which was as well seen on RT-PCR on day 3 (Fig. 5B,C).

On the seventh day of differentiation majority of cells exhibited morphology of fully differentiated neurons, although less than 7% of cells remained rounded without projections and they were positive for nestin. Most of these branched cells were MAP2 (Fig. 4G–I) and β3-tubulin positive and they co-localized with THY1-YPF cells (Fig. 4E–H). The percentage of cells co-expressing THY1-YFP and neuronal markers (MAP2, β3-tubulin) on the seventh day of differentiation was 21.5 ± 0.6% (Fig. 5A). GFAP positive cells represented 3% of a total cell count, and as we expected, they did not co-localize with THY1-YFP positive cells (Fig. 4J–L). Thus expression of Thy1-YFP was observed already in neural stem cells, it was present in 21.88 ± 0.53% of neurons during all stages of differentiation and the signal was present in all parts of the cell.

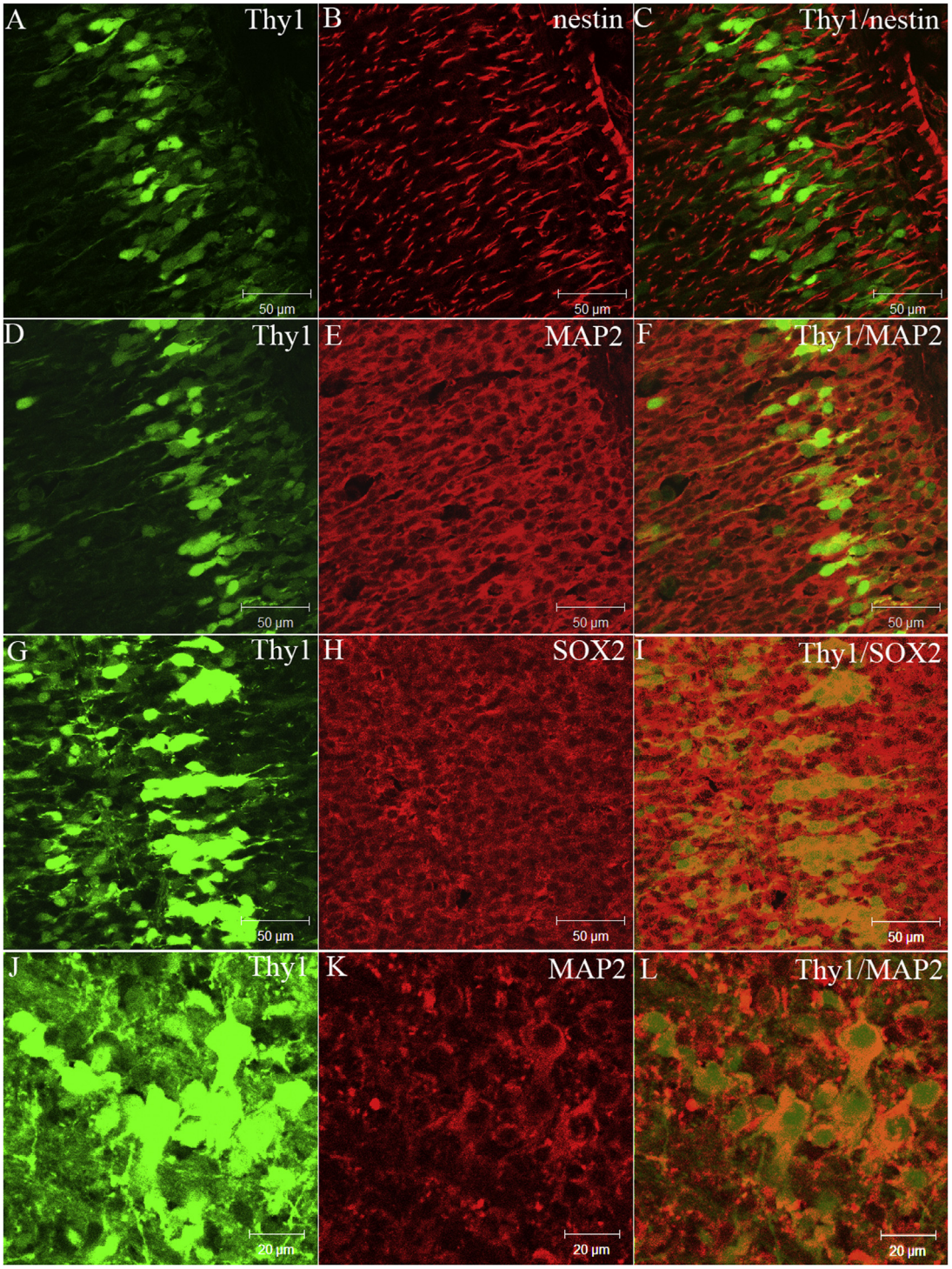

Fig. 2. Thy1-YFP expression during embryonic development. Immunohistochemical reaction against nestin did not reveal co-localization between nestin and Thy1-YFP at E12.5 in the ventral horn of developing spinal cord (A–C). Immunohistochemical reaction against MAP2 (D–F) and SOX2 (G–I) revealed co-localization with Thy1-YFP at E14.5 ventral horn of developing spinal cord. Immunohistochemical reaction against MAP2 (J–L) showed co-localization with Thy1-YFP at E16.5 developing hindbrain.

3.3. Thy1-YFP cells are a robust tool to trace migration, survival and differentiation of stem cells after stereotaxic transplantation in the mouse brain

To analyse to which extent differentiation of transplanted neural stem cells will follow their biological pattern, PKH26 – labelled Thy1-YFP cells were transplanted near hippocampus of the wild type and stroke-affected mice. Brains were isolated 2, 4, 8 and 14 weeks after transplantation.

In the healthy brain two weeks after transplantation cells were present on the site of transplantation and we easily distinguished transplanted cells from the host tissue. A great majority of transplanted cells were clearly PKH26 positive and they exhibited morphology of small rounded cells. A small percentage of these

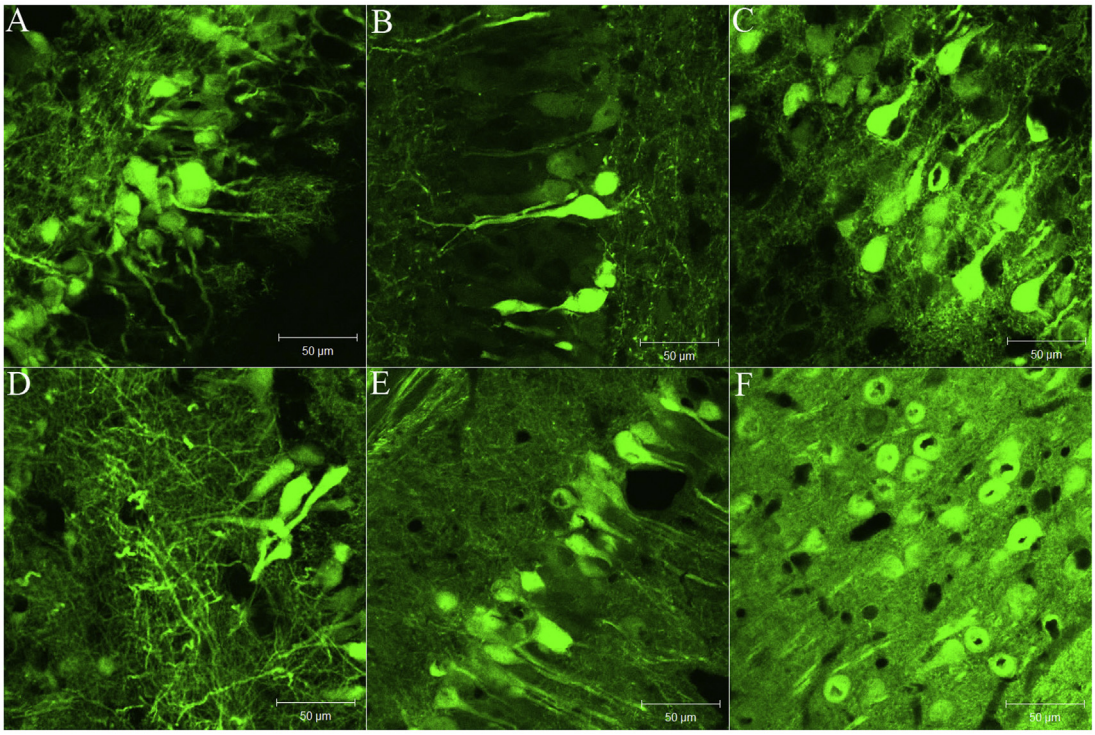

Fig. 3. Thy1-YFP expression in one week (A–C) and one month (D–F) old mouse: olfactory bulb (A and D), hippocampus (B and E) and cortex (C and F).

cells were Thy1-YFP positive and these cells were among those ones which extended projections (Fig. 6A–C). In the stroke-affected brain some cells left the place of transplantation and migrated towards the region affected by ischemia.

In the healthy brain four weeks after transplantation, cells were present on the site of transplantation and some of them migrated down the corpus callosum. Number of Thy1-YFP positive cells was higher than two week after transplantation and those cells revealed more mature morphology (Fig. 6D–F). Because the site of transplantation was near the lateral ventricle some cells entered into ventricle. Those cells which entered ventricle formed spheres which contained more differentiated Thy1-YFP positive cells. Spheres were present in all ventricles, they attached to ependymal cells and their dendrites incorporated into the host tissue. In the stroke-affected brain majority of cells left the place of transplantation and migrated towards the stroke affected region. They appeared as branched neurons and in general, their differentiation reached more mature stage than in healthy brain.

In the healthy brain eight weeks after transplantation intensity of PKH26 dye decreased and only few cells were PKH26 cells positive. On the other hand, Thy1-YFP positive cells were easily visible and they were incorporated into the host tissue (Fig. 6G–I). The main difference between normal and stroke-affected brain was that in normal brain cells made clumps with lower amount of differentiated cells. In the stroke affected brain, differentiated cells exhibited morphology of mature, branched neurons which were completely incorporated into the host tissue.

Fourteen weeks after transplantation, PKH26 positive cells have not been detected into the host brain anymore. On the other hand Thy1-YFP positive cells were clearly visible and they revealed mature phenotype. This was especially clear in the stroke affected brain when they were branched and they connected mutually and with the host cells. Spines on transplanted cells were completely developed and resembled those ones observed in *in vitro* differentiated neurons (Fig. 6J–L).

4. Discussion

Stem cell based therapy holds great promise in finding effective therapeutic strategies for brain diseases [10]. However, more data and knowledge about events occurring after the cells are transplanted in the brain of the patient or experimental animal are needed for follow-up and translation into clinical study. In spite of substantially increased number of experiments based on stem cell transplantation in the last decades, we still lack a satisfactory method to trace cells in a longer time span and to visualize all their projections [3]. So far, any exogenous stain used, including PKH26, which was tested in this work, dilutes and loses its efficiency after 4–6 weeks. Similar problems have been already reported [1]. Moreover we wanted to test to which extent development of cells with fluorescent signal *in vitro* can be compared to that one in *in vivo*.

Results of this study revealed precise pattern of Thy1-YFP expression *in vitro*, during embryonic development and after transplantation into the mouse brain. Although the exact reason why only portion of the cells express Thy1 and why it is present in all parts of the cells remains unknown, we know that Thy1 has widespread functions. They include: inhibition of neurite outgrowth, apoptotic signalling, leukocyte and melanoma cell adhesion and migration, tumor suppression and fibroblast proliferation and migration [11]. Elevated Thy1 expression was described at the site of inflammation, in the formation of the tumour stroma and during tissue remodelling in the case of injuries [12]. Our result that approx. 22% of cells express YFP in analysed line can be explained with mosaicism inherent to this line. On the other hand, this ratio of cells is perfect for precise visualization of all cell processes. If this number would be higher, the signal would be too

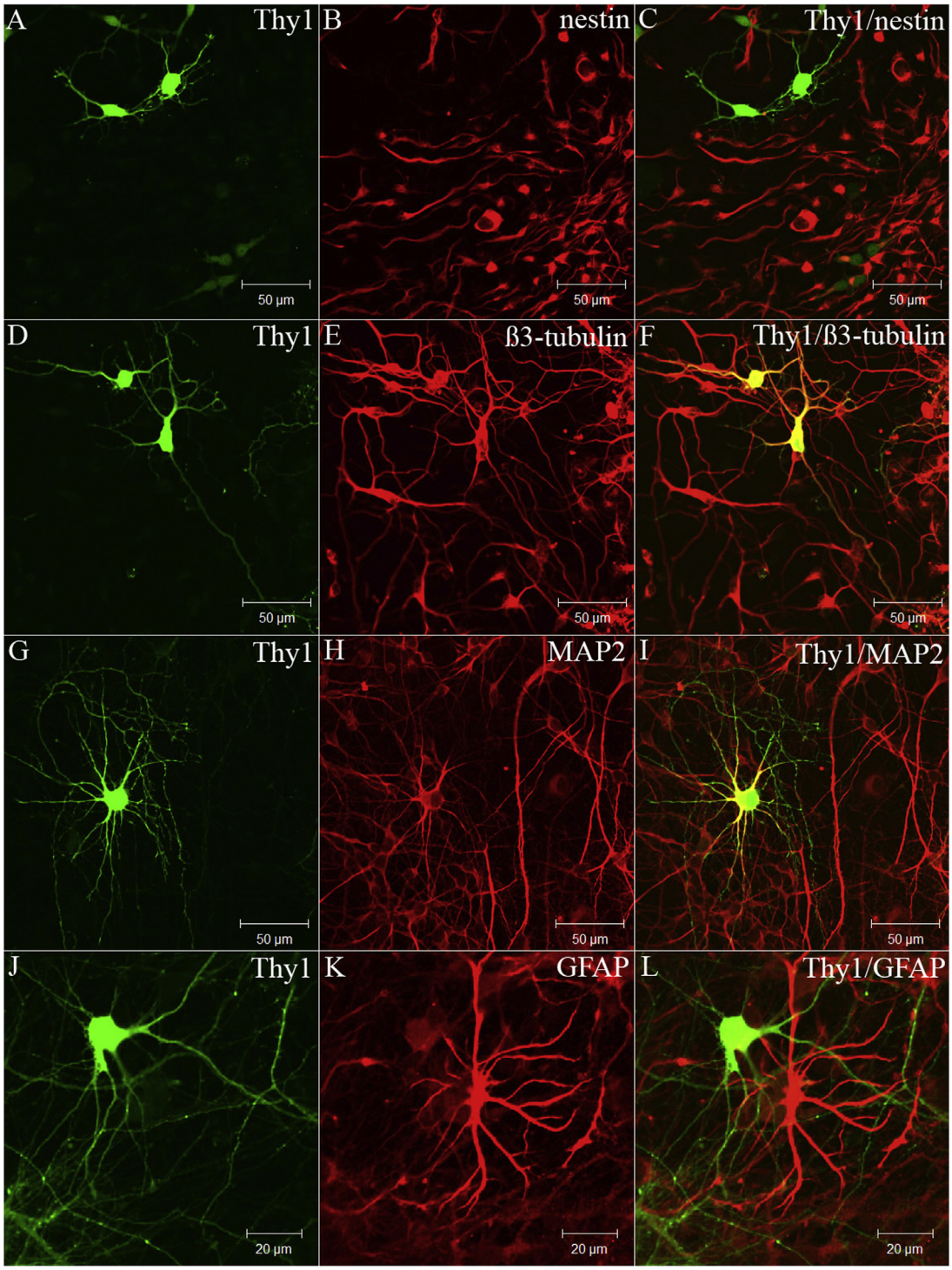

Fig. 4. Expression of Thy1-YFP during differentiation of neural stem cells *in vitro*: Immunocytochemical reaction against nestin on the 1st day of differentiation revealed that Thy1-YFP positive cells in more differentiated stages do not colocalize with nestin, while some immature progenitors exhibit presence of both nestin and weak expression of Thy1 (A–C). Immunocytochemical reaction against β3-tubulin on the 3rd day of differentiation revealed co-localization of Thy1 and β3-tubulin (D–F). Immunocytochemical reaction against MAP2 (G–I) and GFAP (J–L) on the 7th day of differentiation revealed that cells were predominantly neurons, demonstrated with neuron specific markers (MAP2 and β3-tubulin) and they co-localized with Thy1-YFP positive cells. GFAP positive cells did not exhibit co-localization with Thy1.

strong and many processes would be overlapping, thus reducing possibility to trace them precisely.

This work analysed the expression pattern of Thy1 in the strain Thy1 YFP-16. Although it was reported that during development, neuronal expression of Thy1 is low [12] and that undifferentiated neurospheres were not YFP positive [13] in our study we were able to recognize Thy1-YFP positive cells in progenitor cells as well as in mature cells. Progenitor cells were Thy1 positive from the beginning of stem cell isolation and they were nestin and SOX2 positive. Although, Thy1-YFP positive cells, during first five days were not

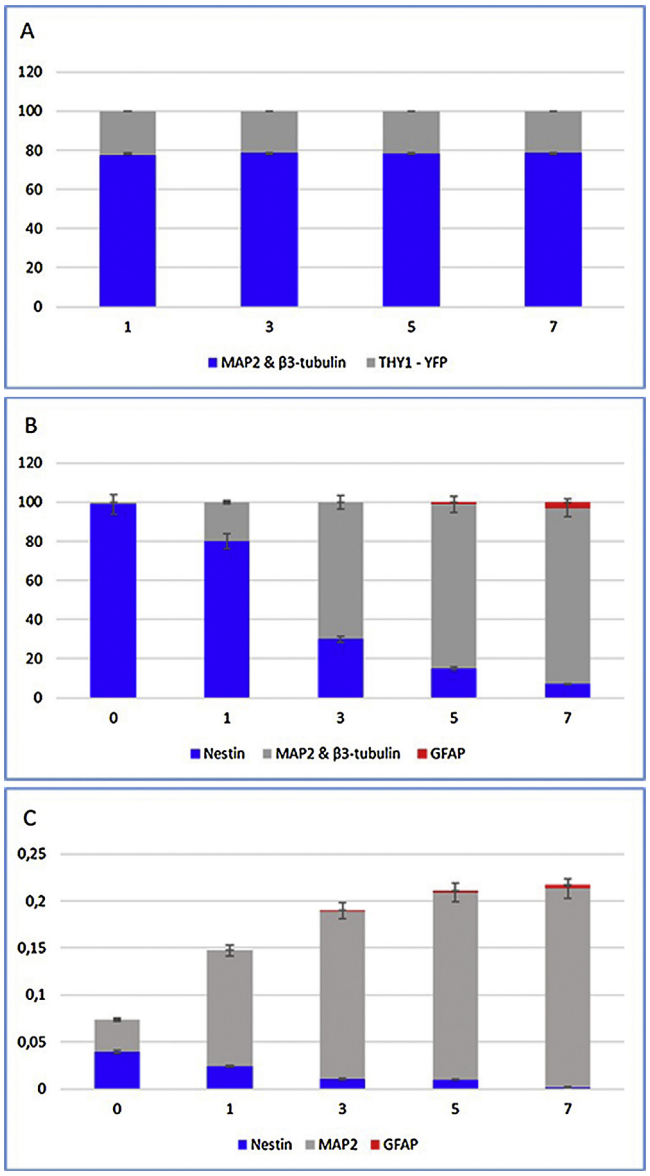

Fig. 5. Quantitative immunocytochemical (A and B) and RT-PCR (C) analysis of differentiation markers in cell culture. A) Number of Thy1-YFP positive cells during *in vitro* differentiation was constant, in the range 21.3–22.5% (average: 21.88 ± 0.53%). B) Number of nestin positive cells during *in vitro* differentiation decreased from 99 to 7%, while number of cells which expressed neuronal markers (MAP2 and β3-tubulin) was constantly increasing. GFAP positive cells were observed on the 5th day of differentiation. C) RT-PCR confirmed results of cell counting. The only difference was that on the 3rd day of differentiation we observed expression of GFAP in a very small subset of cells, which appeared visible on immunocytochemistry two days later. Legend: X – days of differentiation and Y – number of counted cells (A), percentage of counted cells (B) and percentage of RNA expression (C). Bars represent standard deviations.

mature neurons, they expressed typical neuronal markers (MAP2, β3-tubulin and NeuN) but retain SOX2 positivity.

During embryonic development Thy1-YFP positive cells were observed on E12.5 in the central and peripheral nervous system. This is different to Feng et al. [4] who reported that the earliest expression was detectable in motor axons on E13. Moreover, another publication described YPF expression on another, "H" strain and showed that Thy1-YFP expression begins after birth [14]. Another group which used the same "H" strain described YFP fluorescence in cell bodies and axons in pyramidal neurons of the postnatal brain, while on E16 brain, they reported a weak expression only in cell bodies [15]. In this study we described detailed expression of Thy1-YFP during the whole differentiation and subtle differences at various stages. This includes expression in some parts not mentioned before, like in spinal and cranial nerves or in developing teeth on the E14.5.

Complexity of the host nervous tissue and the process of differentiation of the transplanted cells into mature neurons require careful selection of the cell tracing strategy. The oldest and still useful protocols include inter-special transplantations (*e.g.* chicken-quail) or transplantations of male originated cells into female hosts. Introduction of vital dyes or DNA and other cell tracers which bind to certain cell elements (nucleus, cytoplasm or cell membrane) [1,16] offers possibility to easily mark cells and follow them in the limited time period before dyes dilute and cells become phagocyted. Transgenic methods brought possibility to genetically mark cells [4]. If the stable transfection is not possible, then it lasts for limited amount of time. The stable transfections bring either new cell lines or mouse strains with various cell parts and cell subtypes marked.

Although the cell labelling with a specific dye is a relatively simple procedure, this approach has some drawbacks: cell divisions decrease the signal intensity and death – phagocytosis of those cells lead to occurrence of signal in surrounding host cells [1]. Our group has been testing several types of exogenous dyes, including PKH26 (Sigma) or BrdU (Sigma) and all these methods are reliable but only for short to mid-term tracing applications (up to 4 weeks). Since ubiquitous fluorescent marker – expressing transgenic strains fail to mark all cell projections, which we as well described in our own works [3] here we suggest a use of Thy1-YFP strain for several applications not been described so far: apart from analyses of neuronal differentiation during embryo development, neural stem cells isolated from this strain can be successfully used in *in vitro* experiments, where this line offer possibility to visualize every single cell projection, and in studies when long tracing of transplanted cells with neuronal fate is needed. Reported fact that 21.88 ± 0.53% of neurons obtained from this strain express Thy1-YFP is ideal ratio for visualizing all processes, which is comparable to Golgi staining. Stability of the signal and even its increased presence in stroke, which is similar to some already reported genes [17], confirms high applicability of this model. Moreover this strain has been recently rediscovered by several groups suggesting application in some specific fields of interest: visualization of neuromuscular junction [18], organotypic culture of retina [19] or even for visualizing experimental tumours, inflammation and wound-healing [20].

5. Conclusions

Our results showed that, in addition to mature neurons, neuronal progenitor cells express Thy1-YFP both in *in vivo* and *in vitro* conditions. This suggests that in addition to analyses of neuronal differentiation in Thy1 YFP-16 mouse, neural stem cells isolated from this strain can be successfully used for long term tracing of cells in which analyses of neuronal fate is needed. This has been confirmed in both healthy and stroke affected mouse brain.

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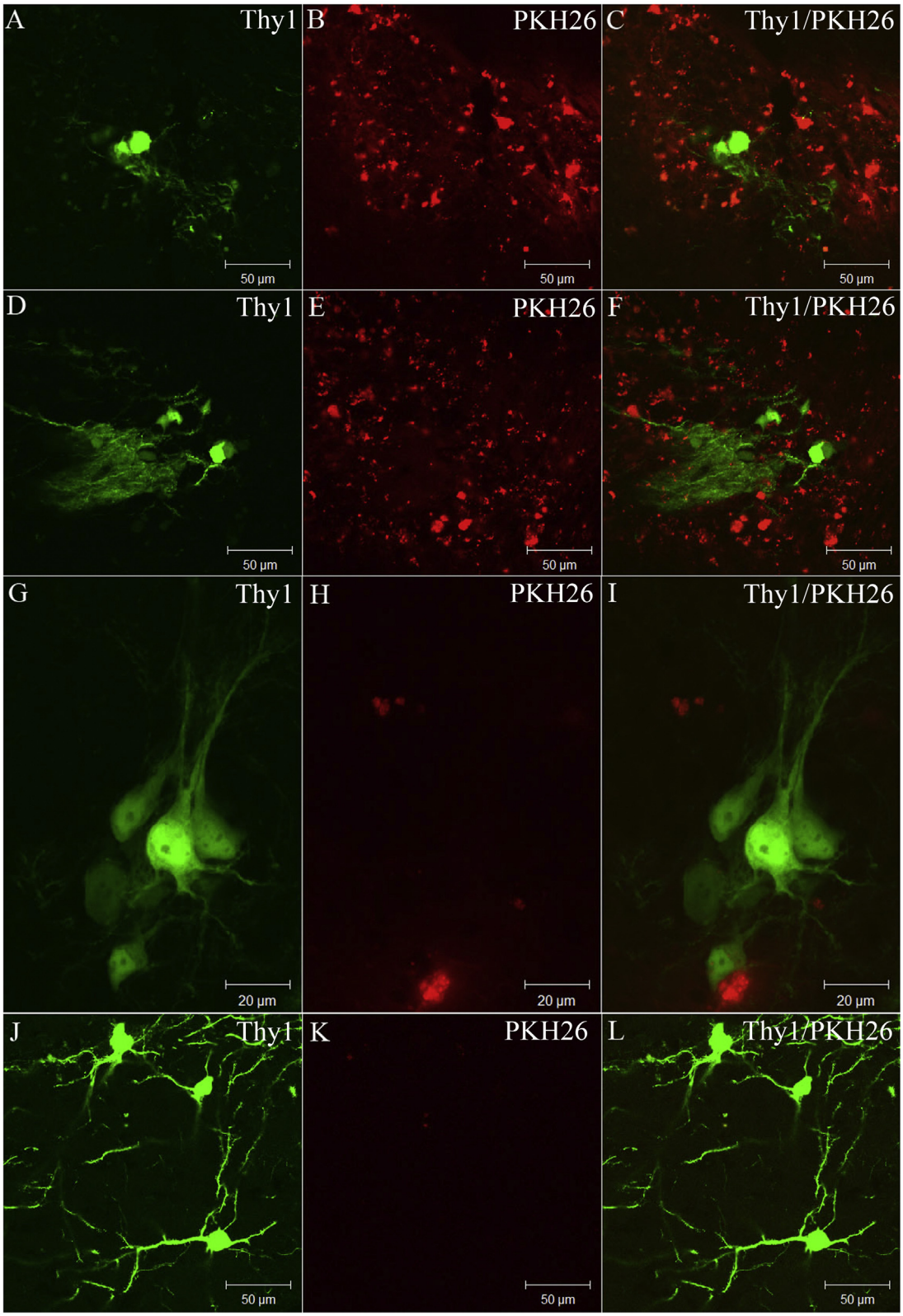

Fig. 6. Thy1-YFP expression after transplantation of PKH26 labelled cells in the mouse brain after 2 weeks (A–C), four weeks (D–F), eight weeks (G–I) and fourteen weeks (J–L). Intensity of PKH26 after four weeks diluted and it was lost, while expression of Thy1-YFP positive cells increased in all part of neurons.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2016.10.001.

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