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PRINCIPAL INVESTIGATOR: Patrik Brundin, M.D., Ph.D.

CONTRACTING ORGANIZATION: Lund University
Wallenberg Neuroscience Center
SE-221 82 Lund, Sweden

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Patrik Brundin, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lund University
Wallenberg Neuroscience Center
SE-221 82 Lund, Sweden

E-Mail: Patrik.Brundin@neuro.lu.se

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13. ABSTRACT (Maximum 200 Words)

The project aims to differentiate human embryonic stem cells (hESCs) into dopaminergic neurons for use in neural grafting in Parkinson's disease (PD). During the first year we studied the survival and differentiation potential of hESCs implanted into a rat model of PD. hESCs were differentiated on PA6 feeders for 16, 20 and 23 days prior to grafting. The number of neurons and dopaminergic cells increased with time in vitro. 100,000 viable cells were grafted into the striatum of immunosuppressed 6-OHDA-lesioned rats. The grafted hESCs-derived cells survived well in all groups. Substantial number of surviving cells differentiated into neurons (NeuN positive) but very few hESC-derived TH positive neurons were observed in most transplanted rats. Amphetamine-induced rotational behavior was tested at weeks 2, 4, 8 and 13 after transplantation. No behavioral recovery was observed. Importantly, the rats grafted with hESCs differentiated for 16 days developed severe teratomas staring from 6 weeks post-transplantation, while most rats grafted with hESCs differentiated in vitro for longer periods kept healthy until the end the experiment. This indicates that differentiated hESCs can survive and retain neuronal phenotype after transplantation despite low numbers of dopaminergic neurons and that the differentiation of pre-transplantation is essential to prevent teratoma-formation.

14. SUBJECT TERMS
Parkinson's disease, transplantation, embryonic stem cell, neuronal differentiation, dopamine, xenograft, functional recovery

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Introduction

Grafting of primary embryonic neural tissue has proven that cell replacement therapy is a viable approach to the treatment of Parkinson's disease (PD). However, the number of successfully grafted patients is relatively small, partly due to limited access to suitable donor tissue. Alternative sources of donor tissue are urgently needed for cell transplantation to become a widely available therapy for PD. Human embryonic stem cells (hESC) have been suggested to be a potential tissue source for cell replacement therapy in PD. They can be differentiated into a variety of cell types including neurons (Stojkovic et al., 2004). Regarding differentiation into dopaminergic neurons, previous studies showed that mouse ESC can indeed differentiate into this cell type under certain culture conditions (Kawasaki et al., 2000; Lee et al., 2000; Wakayama et al., 2001; Kim et al., 2002; Barberi et al., 2003). Cells derived from mouse ESC can also survive transplantation into rodent brains (Kawasaki et al., 2000; Bjorklund et al., 2002; Kim et al., 2002; Morizane et al., 2002; Nishimura et al., 2003). When non-differentiated mouse ESCs were grafted into the striatum, substantial numbers of mouse ESC differentiated into dopamine (DA) neurons. However, many grafted rats developed teratomas (Bjorklund et al., 2002). If mouse ESC were differentiated into neurons *in vitro* prior to transplantation, much higher numbers of DA neurons were observed after grafting. There was convincing evidence for functional recovery, in terms of improvement of lesion-induced behavioral deficits, without the formation of teratomas (Kawasaki et al., 2000; Kim et al., 2002; Barberi et al., 2003). Similar results have also reported for monkey ESC (Kawasaki et al., 2002; Takagi et al., 2005).

In the present project, we proposed 1) to expand cells from the established hESC line (SA002) and to identify epigenetic stimuli that promote their differentiation into a uniform, enriched population of DAergic neurons; 2) to assess the DAergic phenotype of the hESC-derived neurons ***in vitro*** and 3) to assess the ability of grafted hESC-derived neurons to restore DAergic neurotransmission ***in vivo.***

Body

During the first year, we have made considerable progresses in the proposed project. Unfortunately the Gothenburg team failed to recruit a post doctoral fellow.

In vitro neuronal induction and DAergic differentiation:

We demonstrated that hESCs from the line SA002 developed the highest number of tyrosine hydroxylase- (TH, a DA synthesizing enzyme) and βIII tubulin- (a marker for neurons) positive colonies already after 2.5 weeks in culture (38,8 ± 22,8 and 42,5 ± 20, respectively, n = 81). Immunostaining for peripherin, a marker for neurons of the peripheral nervous system, showed that the vast majority of TH-immunoreactive neurons had a phenotype consistent with *central* nervous system neurons. Thus, only 1.0 ± 2.0 % of the TH-immunoreactive cells stained also positive for peripherin after 6 weeks in culture.

In vivo transplantation:

The hESCs were induced to undergo differentiation by being grown on PA6 stromal cell feeders for 16, 20 or 23 days. The numbers of neurons (βIII tubulin positive) and specifically dopaminergic (TH positive) neurons increased with time in culture. These encouraging results obtained *in vitro*, prompted us to perform neural transplantation studies. Forty rats were first unilaterally injected with 6-OHDA to the medial forebrain bundle to generate rats with a unilateral Parkinsonian syndrome. The animals were then subjected to a test of motor asymmetry ("rotation testing") after administration of amphetamine. Based on the rotation scores and our prior experience of this animal model, we identified 38 of the 40 rats as having complete (> 98% DA depletion) lesions of the nigrostriatal pathway. Since such animals do not display spontaneous recovery of functions, they were selected to undergo cell transplantation.. The rats were divided into 3 groups and intrastriatally implanted with 100,000 viable hESCs-derived cells that had been cultured for 16, 20 and 23 days, respectively (referred as Group 1, Group 2 and Group 3 accordingly in the Report) (Fig. 1). All rats were immunosuppressed with cyclosporine A (15mg/kg/day, i.p.), which has previously been shown to suppress immune rejection of intracerebral xenografts. Motor asymmetry was

assessed again at 2, 4, 8 and 13 weeks after implantation and the scores were compared to pre-grafting values

Behavioral tests:

At the amphetamine-induced rotation tests performed after grafting, there were no significant changes in rotation scores suggesting that the grafts had induced behavioral recovery (Fig. 2)

Characterization of grafted cells:

In order to assess the degree of differentiation (numbers of βIII tubulin- and TH-positive cells) of the cells we grafted, we plated some PA6 cells induced hESCs on polyornitine and laminin coated glass chamber slides before transplantation and allow these cells to be maturely differentiated. In addition, at the end of transplantation operation, the some cells that were not implanted were plated on coated culture dish only for 24 hours and fixed for immunocytochemistry (Fig. 3). Large numbers of grafted hESC-derived cells were positive for βIII tubulin in all 3 groups (Fig. 4). The number of βIII tubulin increased with time spent growing on PA6 feeder cells. Up to 30 % of the total number of cells were positive for βIII tubulin after 20 days in co-culture (Fig. 4A and Fig. 5b). More importantly, differentiation into TH-positive neurons was also enhanced with time from 16 to 23 days. After 23 days, 35% of the βIII tubulin-positive cells were also positive for TH (Group3) (Fig. 5a). Consequently, up to 7% of the total number cells were positive for TH (Fig. 5c). In summary, these data indicate that neuronal and DAergic differentiation is enhanced when hESCs are co-cultured with PA6 stromal cells for a long periods, and that differentiation is further promoted also between 16 and 23 days after plating the hESC cells on the PA6 feeder cells.

Cell survival:

Rats from each group selected and killed at week 2, 6 and 13 after transplantation surgery. We first assessed the rate of survived grafts by examining human nuclear marker positive cells. At week 2, substantial numbers of surviving grafted cells were identified. Stereological analysis showed that total numbers of human nuclear marker-positive cells

were even higher than the numbers of originally grafted cells in Group 1 and 2, indicating continued cell proliferation (see below) (Fig. 6 and Fig. 7)

Characterization of grafted hESCs-derived cells:

A number of cellular and tissue markers were used to characterize the phenotypic differentiation of the implanted cells. First, we examined neuronal differentiation with NeuN, a marker for mature neurons. Large numbers of human nuclear marker-positive cells were also positive for NeuN (Fig. 8), reflecting that the grafted cells had maintained a neuronal phenotype that they already had at the time of grafting, or had matured into neurons following injection into the adult host brain. When comparing the numbers of surviving neurons between the different groups at week 2, the highest proportion was seen in Group 1 (up to 70% of the human nuclear antigen positive cells coexpressed NeuN) whereas it was much lower (32%) in Group 3 (Fig. 9). This tentatively suggests that more mature and fully differentiated neurons obtained after long periods of cultivation *in vitro* survive transplantation poorly. Furthermore, only very few hESC-derived DAergic neurons (around 10-50 TH-positive cells per rat brain) survived in all three groups (Fig. 10). In addition to evaluating neuronal phenotype in the grafted cells, we used the astrocytic marker, S100b to examine glial differentiation. Aside from a low number of cells in rats from Group 3 (23 days of PA6 cell co-culture), we did not observe any hESC-derived astrocytes (Fig. 11).

Continues proliferation of grafted cell and teratomas formation:

Grafting undifferentiated hESCs is, by nature of their pluripotency, associated with high risks of teratoma formation. In order to access to what extent there was ongoing cell proliferation at the time of killing the rats, we administered BrdU (50 mg/kg, i.p.) to each rat 3 times at 8 hours intervals, starting 24 hours before sacrifice. Double staining with human nuclear marker and BrdU demonstrated that substantial numbers of grafted cells were still proliferating (labeled positive for both markers), particularly in Group 1 (Fig. 12). By comparison, in Groups 2 and 3, the rate of proliferation had declined markedly (Fig. 13). This suggests that the proliferative capacity of hESC declines dramatically between 16 and 23 days in vitro when plated on PA6 cells. However, the

existence of dividing cells in grafts obtained from 23 days old cultures, indicates that the cells are still not all uniformly undergoing differentiation after this length of time in culture.

Six weeks after transplantation, we observed a dramatic difference in teratoma formation between the 3 groups. Rats in Group 1 developed large teratomas 6 weeks post-grafting. All the rats in this group gradually appeared to get sick (stopped feeding and grooming and appeared apathetic) and were therefore killed within 11 weeks. They were all found to have large tumerous growths in the grafted striatum, and this was the likely cause of their malaise. Macro- and microscopic images demonstrated typical features of teratomas (Fig. 14). In contrast, most rats grafted with cells that had differentiated *in vitro* for a longer period (Groups 2 and 3) stayed healthy to the end point of the experiment (week 13). Some rats that did not develop large tumor-like teratomas macroscopically also expressed endodermal and mesodermal markers (Fig. 15). The data indicate an inverse correlation between the rate of teratoma formation and the extent of *in vitro* differentiation, i.e. the longer in vitro differentiation the less risk of teratoma formation. Therefore we conclude that it is essential for hESCs to be differentiated *in vitro* prior to grafting to avoid possible tumor formation. In addition, we believe that we may have identified that under the present culture conditions 20-23 days is an adequate time to allow sufficient maturation of the cells such that they are unlikely to form true teratomas. However, the existence of dividing cells and the rare occurrence of cells expressing mesodermal markers also in the rats in Group 3 are warning signs that not even 23 days under the present culture conditions is a sufficiently long time to ensure that there is no risk of teratomas.

Key Research accomplishments in the first year

1. Characterization in vitro differentiation of hESC line (SA002) into DAergic neurons.
2. Assessment of survival of hESC-derived cells after implantation to the striatum in 6-OHDA lesioned rats.
3. Detailed morphological characterization of neuronal and DAergic differentiation of grafted hESC-derived cells.

4. Demonstration of close inverse correlation between degree of *in vitro* differentiation and extent of *in vivo* teratoma formation after grafting

Reportable outcomes

Presentations:

Jia-Yi Li "Stem cell therapy for neurodegenerative diseases: where do we stand now" in 10th annual Meeting of International Society for Cell Therapy, May 7-10, 2004

Jia-Yi Li, Stem cell therapy for Parkinson's disease: The challenges facing us" in "Stem Cells – from progress to therapy?" in Edinburgh, March 3-5, 2005

Peter Eriksson: Invited speaker at Nikko International Symposium, Nikko, Japan 25 September 2004. "Stem cells: tools and targets for brain repair."

Abstracts:

Sofia Correia, Anke Brederlau, Sergey Anisimov, Gesine Paul, Laurent Roybon, Peter Eriksson, Patrik Brundin, Jia-Yi Li: submitted abstract, 3rd Annual Meeting of the International Society for Stem Cell Research June 23-25, 2005 San Francisco, CA USA. "Developing human embryonic stem cell-based therapy for Parkinson's disease"

Manuscripts:

Laurent Roybon, Patrik Brundin and Jia-Yi Li. Stromal cell-derived inducing activity does not promote dopaminergic differentiation, but enhances differentiation and proliferation of neural stem cell-derived astrocytes. Experimental Neurology, Under revision.

Funding applied for based on work supported by this award:

Marie Curie Incoming International fellowship contract MIF1-CT-2005-008445 (appr US$ 381998).

Konung Gustaf V:s och Drttning Victorias Stiftelse (appr US$ 70 761)

Scientific methods:

Improved protocols of in vitro differentiation of hESCs

Conclusions

During the first year of this project we have demonstrated substantial number of surviving hESC-derived neurons (NeuN positive) after intrastriatal implantation, while a few hESC-derived TH positive neurons are observed in the transplanted rats. Most importantly, we demonstrated an inverse correlation between in vitro differentiation and formation of teratomas after grafting and may identify a window of opportunity to transplant hESC-derived cells without the risk of tumor formation.

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Appendices

All the figures of the report are placed in the appendices.

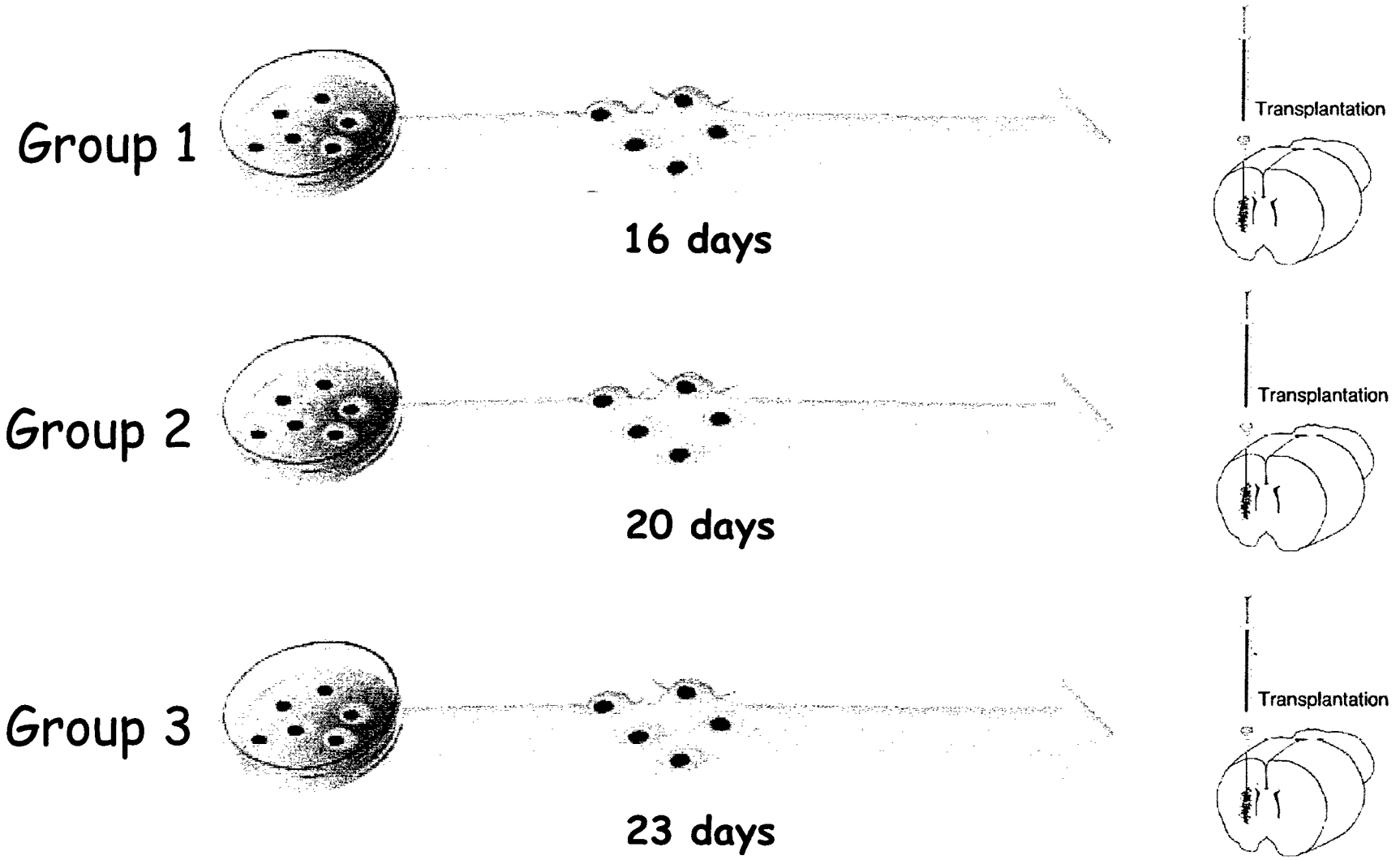


Fig. 1. Transplantation of initially differentiated hESCs for three different time points in co-culture with PA6 cells. Rats in Group 1 were transplanted with cells cultured for 16 days, Group 2 for 20 days and Group 3 for 23 days.

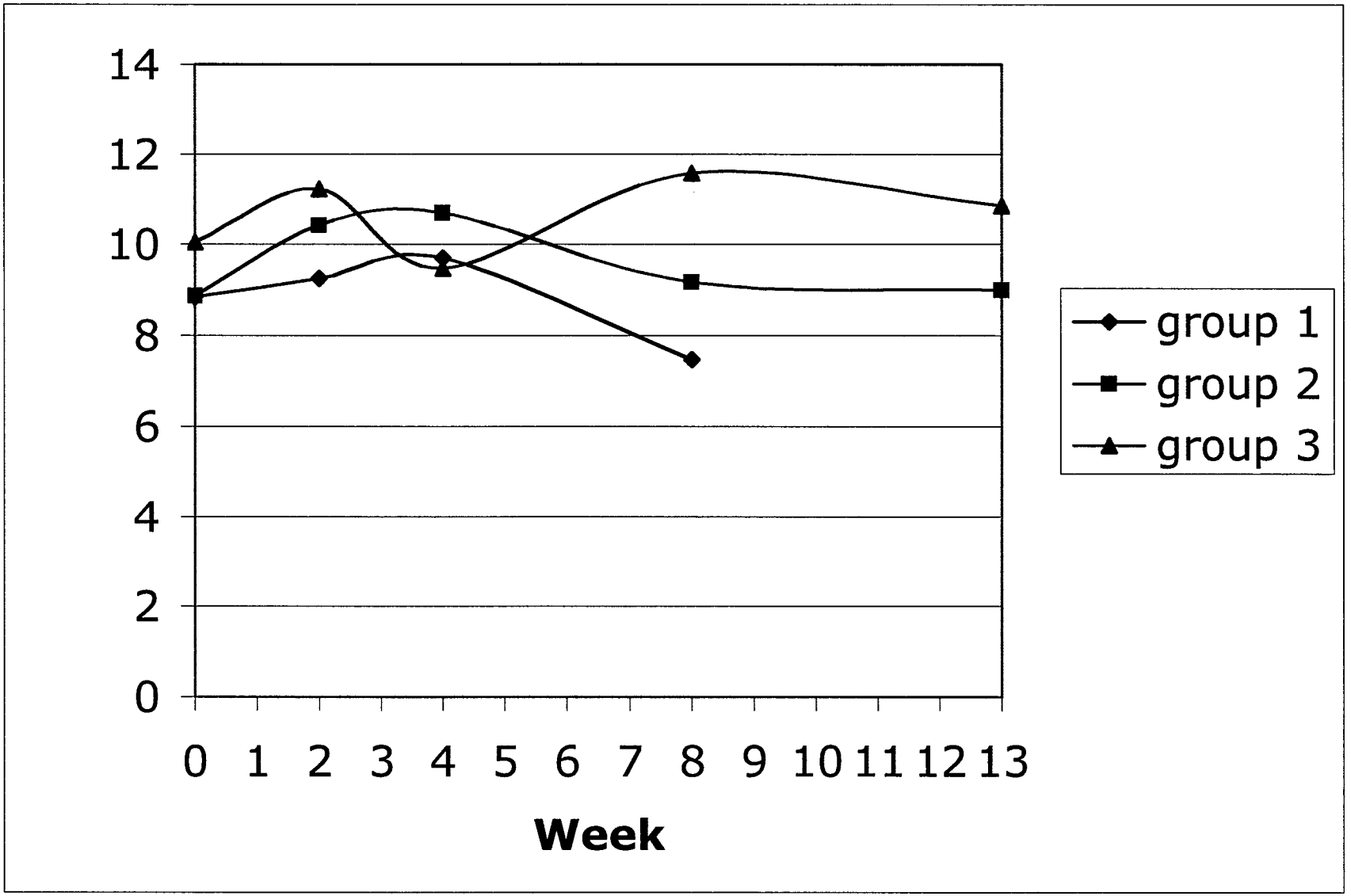


Fig. 2. Amphetamine-induced rotation tests at pregrafting (point 0) and at 2, 4, 8 and 13 weeks after implantation of hESC-derived cells. There is no clear recovery of rotation scores in all 3 groups.

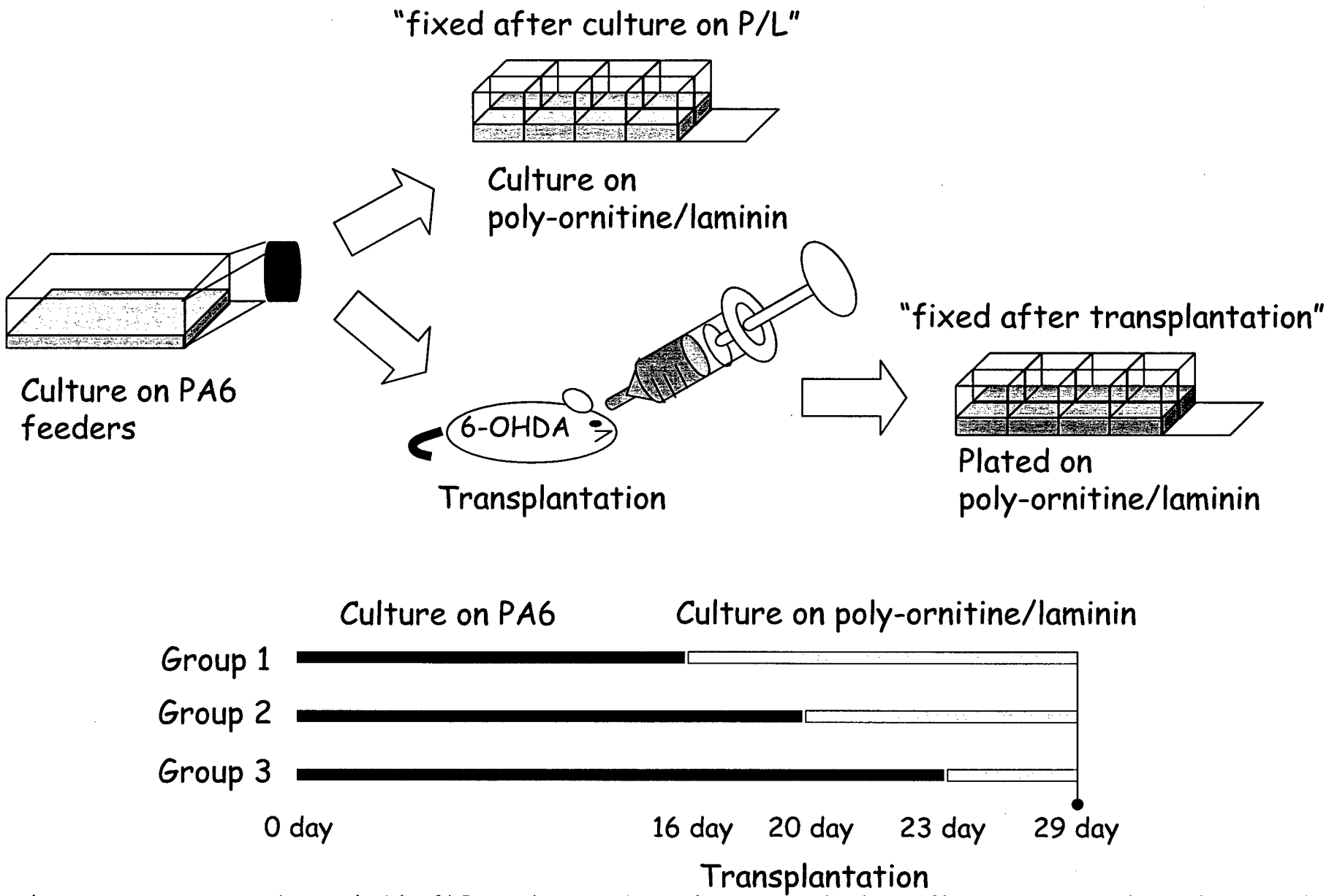


Fig. 3. Schematic representation and time schedule of hESCs culturing and transplantation. At the three different time points of transplantation (blue bars) cells were harvested to be transplanted and some cells to be cultured on poly-ornitine/laminin (P/L) coated chamber slides until a total culturing time of 29 days (yellow bars). These cultures on chamber slides were called "fixed after culture on P/L". The spared cells after transplantation were also plated on P/L coated chamber slides and fixed 24 hours later. These last cultures were called "fixed after transplantation".

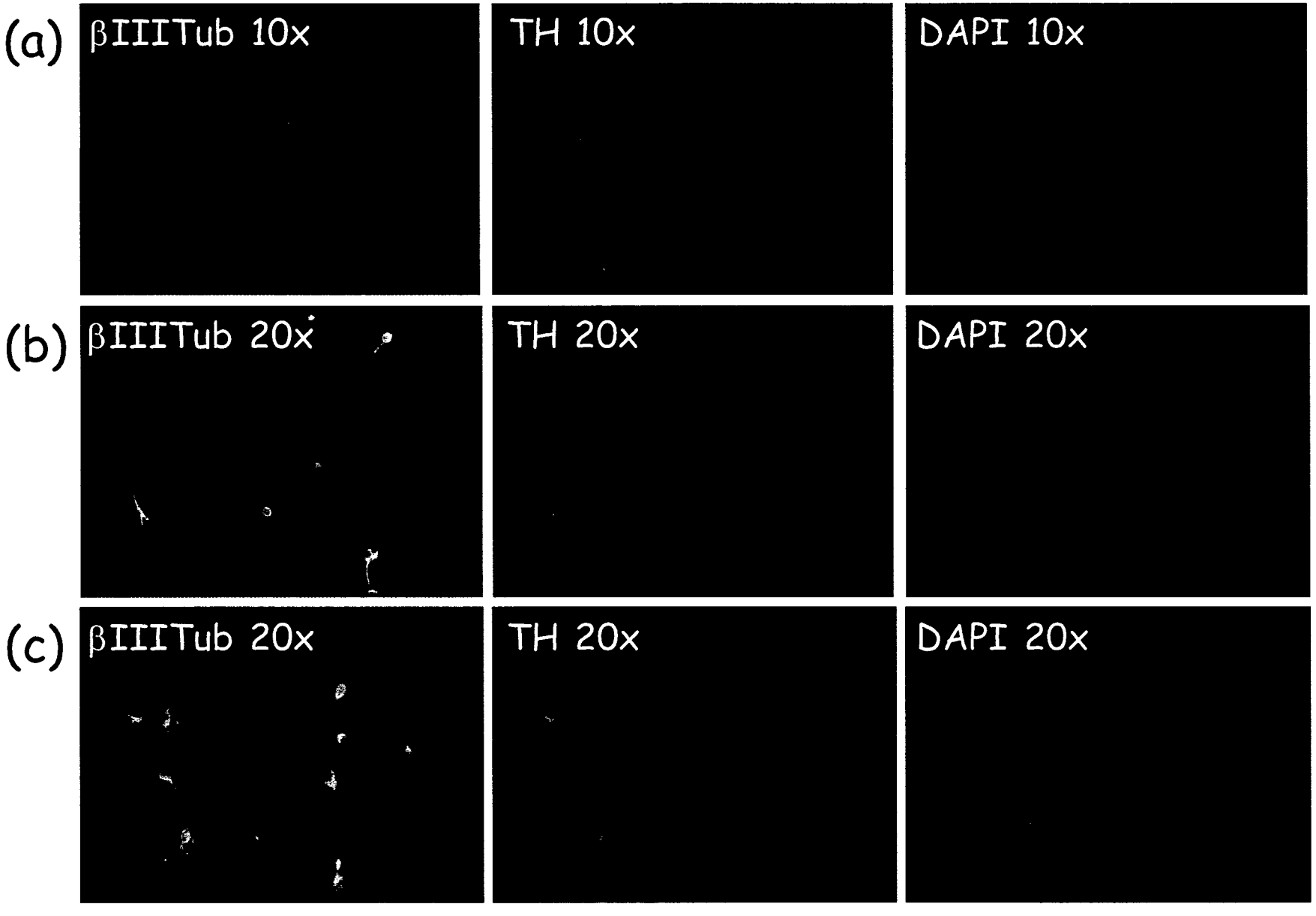


Fig. 4. Immunohistochemical analysis of cells on chamber slices "fixed after transplantation". (a) Group 1, (b) Group 2 and (c) Group 3. Neuronal cells were identified positive for βIII Tubulin. Tyrosin hydroxylase (TH) was used as a marker for dopaminergic neurons. Total cells were visualized by DAPI nuclear staining.

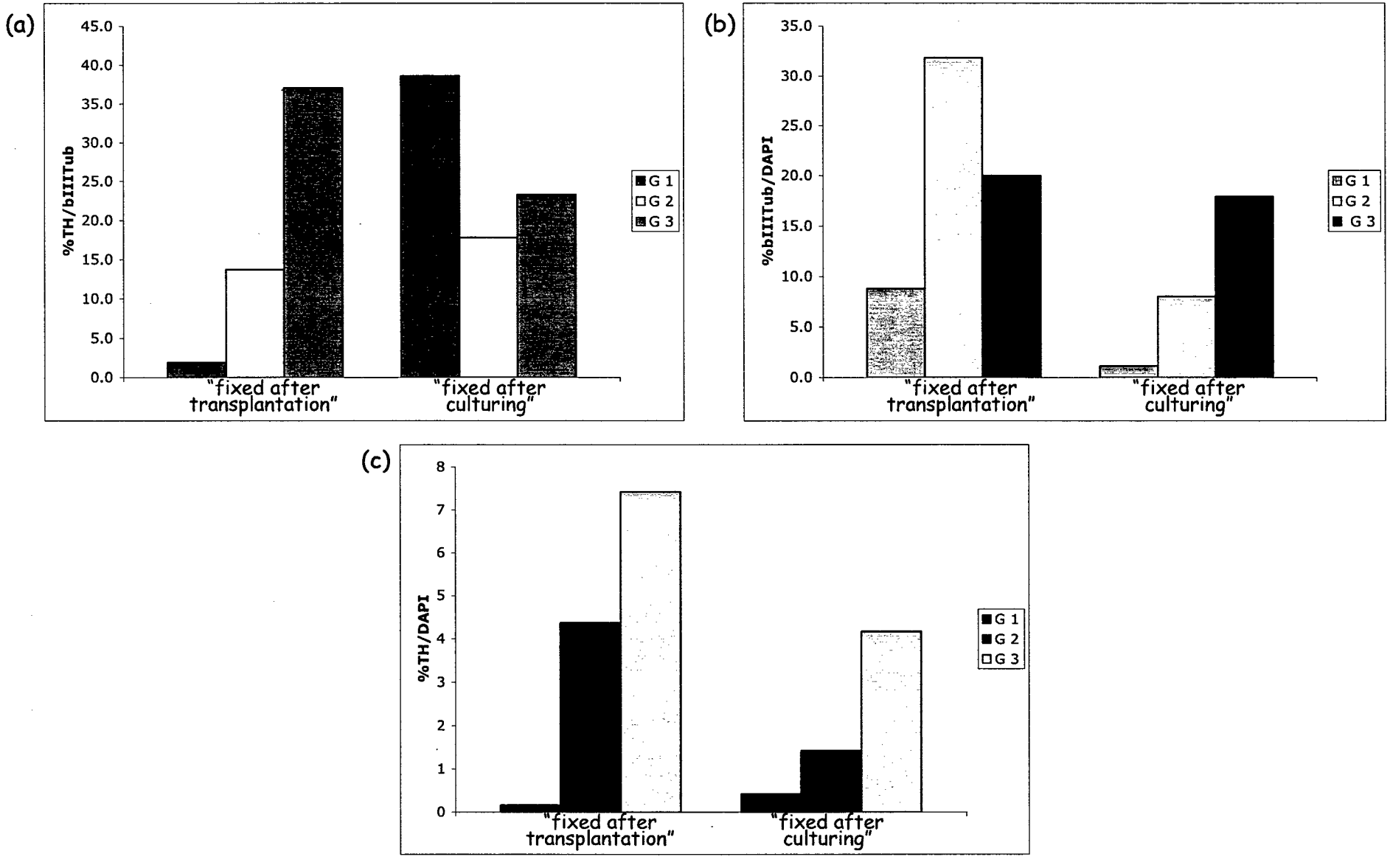


Fig. 5. Quantitative analysis of βIII Tubulin- and TH-positive cells in grafted cell populations. (a) Percentage of TH-positive cells in the total number of βIII Tubulin-positive cells. (b) Percentage of βIII Tubulin -positive cells in the total number of cells marked by DAPI. (c) Percentage of TH-positive cells in the total number of cells.

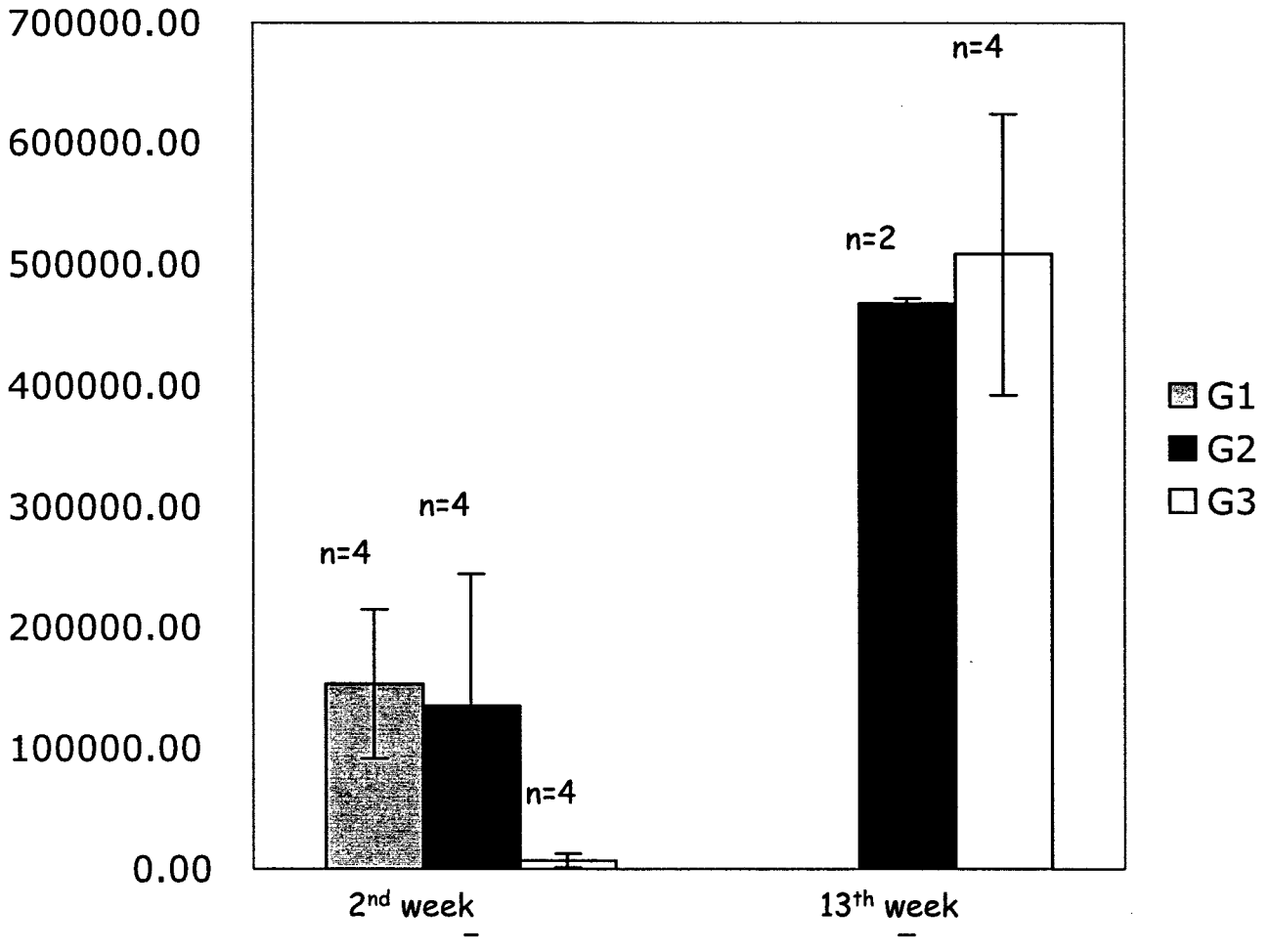


Fig. 6. Stereological analysis of the survived cells per graft dentified by immunostaining with human nuclear marker (HNuc). The mean value between a "n" number of animals which is written on top of each bar. Only brains not showing teratoma formation perfused at the 2nd week and at the 13th week after transplantation were counted.

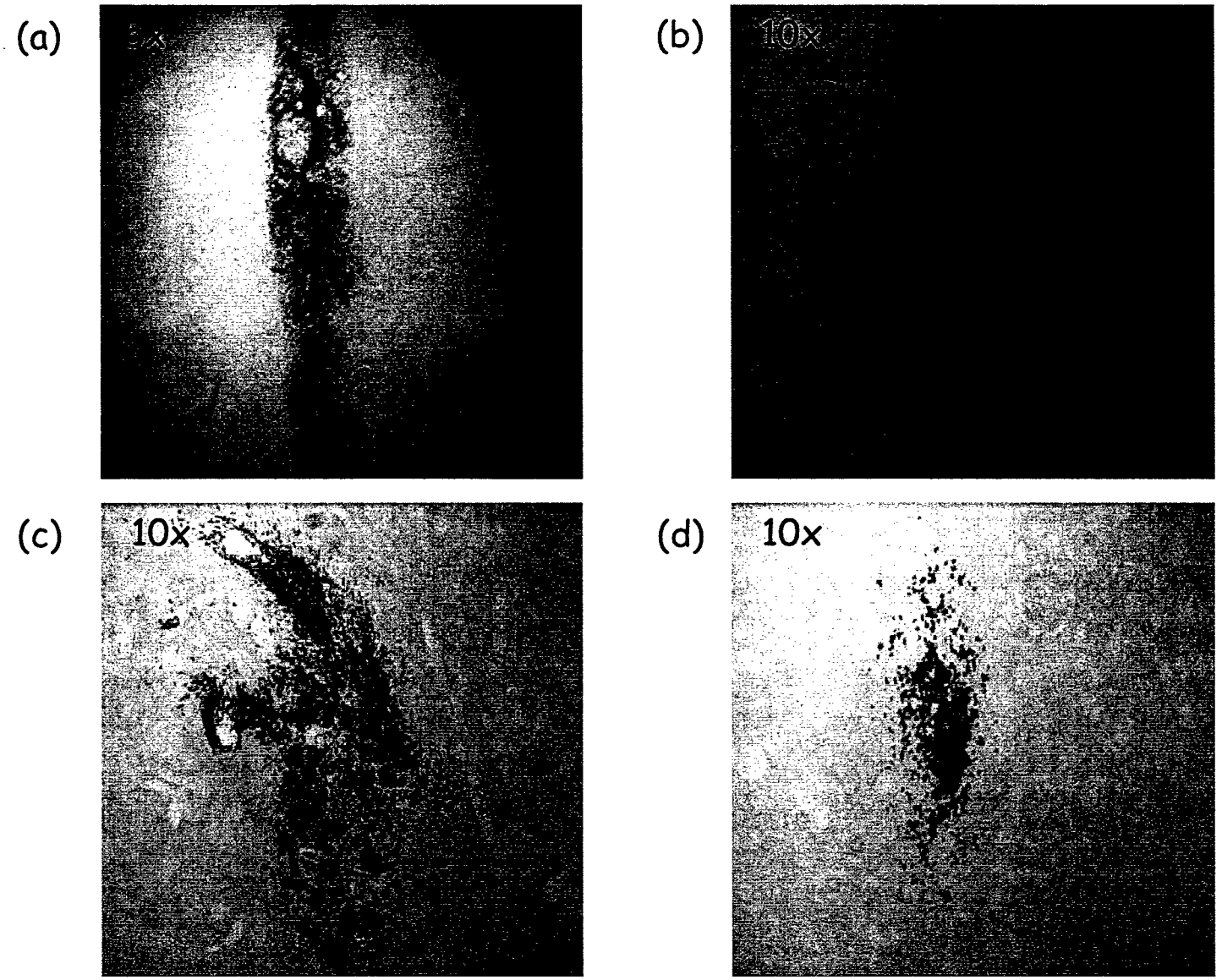


Fig. 7. Human nuclear marker immunostaining from the three different groups perfused 2 weeks after transplantation. (a) and (b) graft from Group 1 at to different magnifications. (c) graft from Group 2. (d) graft from Group 3.

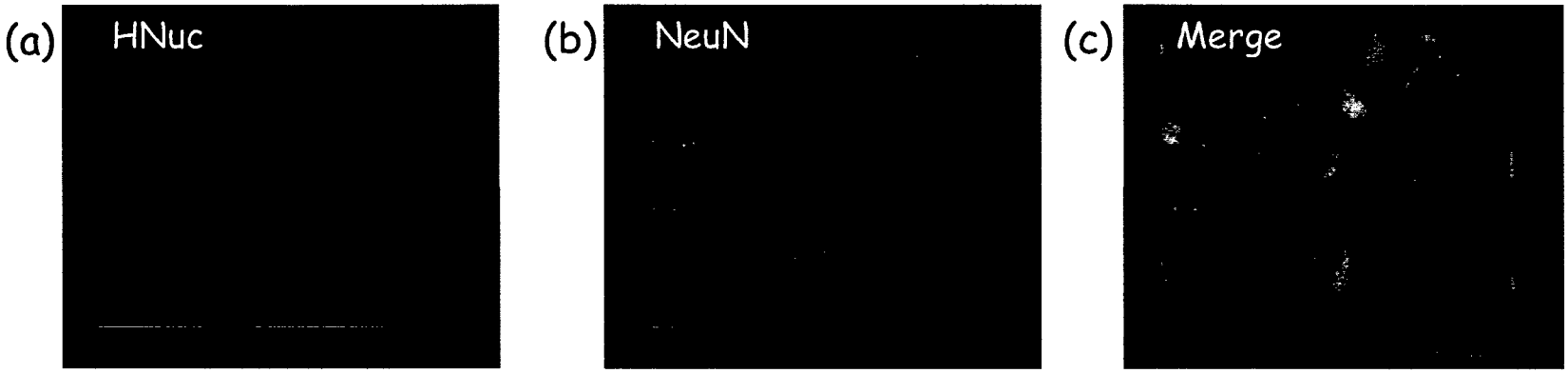


Fig. 8. Identification of neuronal cells within the grafts by double immunostaining for human nuclear marker (HNuc) and neuronal nuclei (NeuN). Confocal images of (a) positive cells for HNuc, (b) for NeuN, (c) double positive cells in yellow.

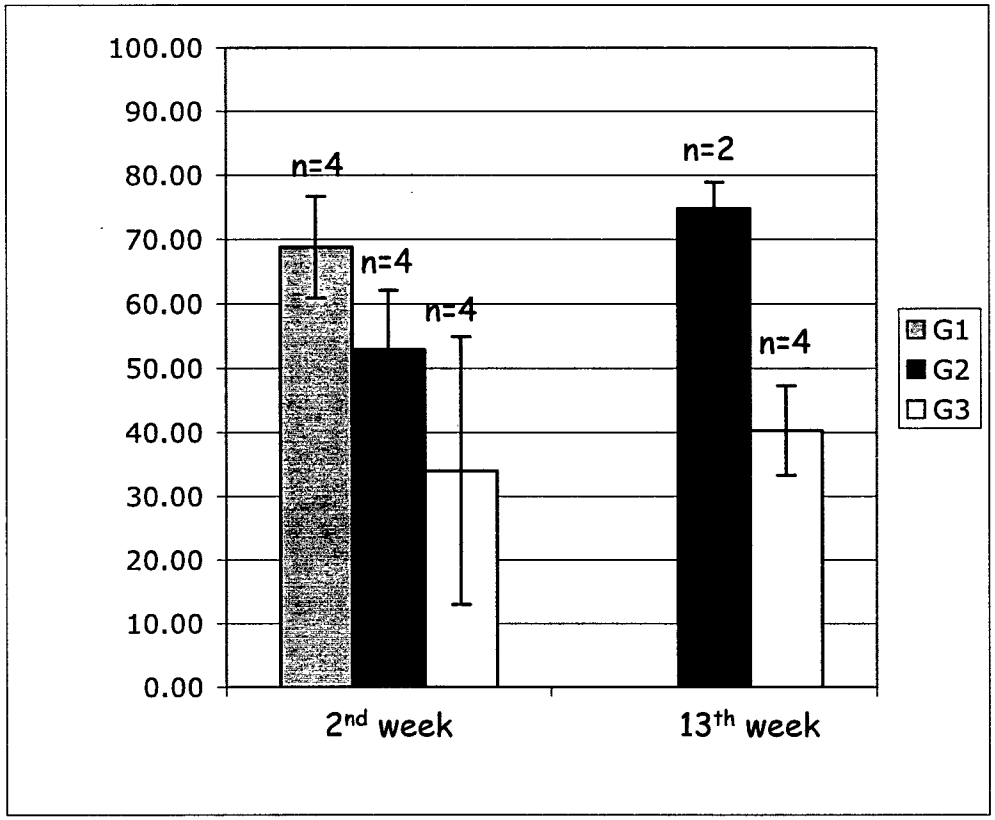
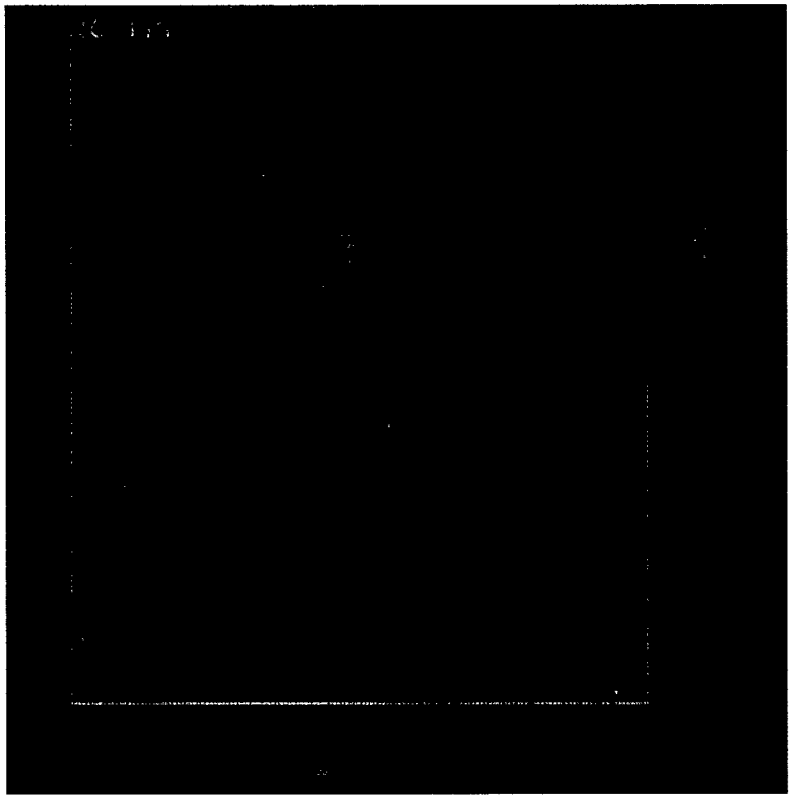


Fig. 9. Stereological analysis of the neuronal cells within the grafts identified by double immunostaining for human nuclear marker (Hnuc) and neuronal nuclei (NeuN). Only brains not showing teratoma formation are included at the 2nd week and at the 13th week after transplantation.

(a)



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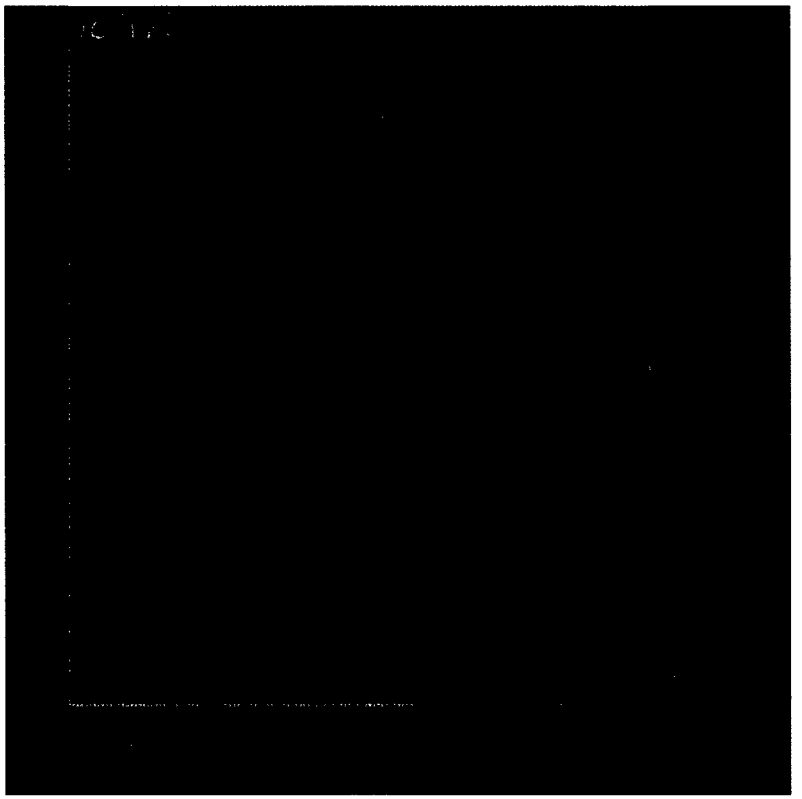

Fig. 10. Identification of hESC-derived dopaminergic neurons within the grafts by double immunostaining for human nuclear marker (HNuc) and tyrosine hydroxylase (TH). (a) and (b) are confocal images of two double positive cells.

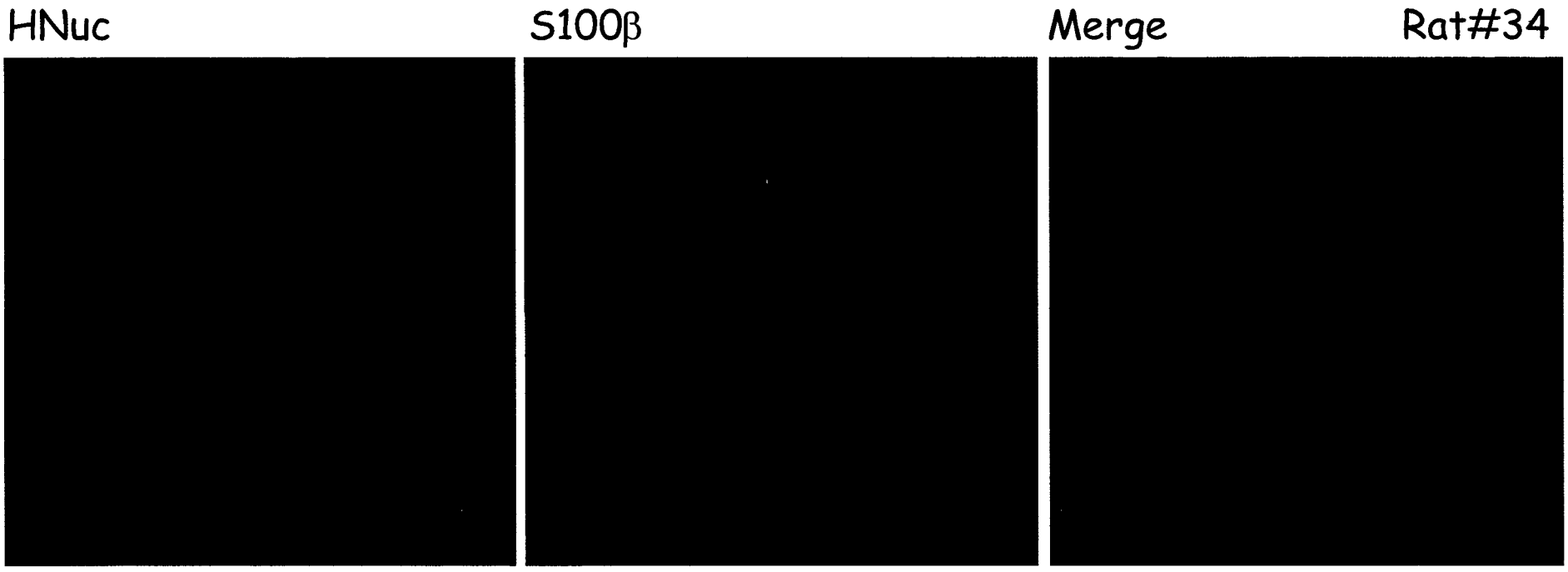

Fig. 11. HESC-derived astrocytes within the grafts in group 3 identified by double immunostaining for human nuclear marker (HNuc) and S100β. (a) HNuc positive cells. (b) S100β positive cells. (c) mergde image with double positive cells.

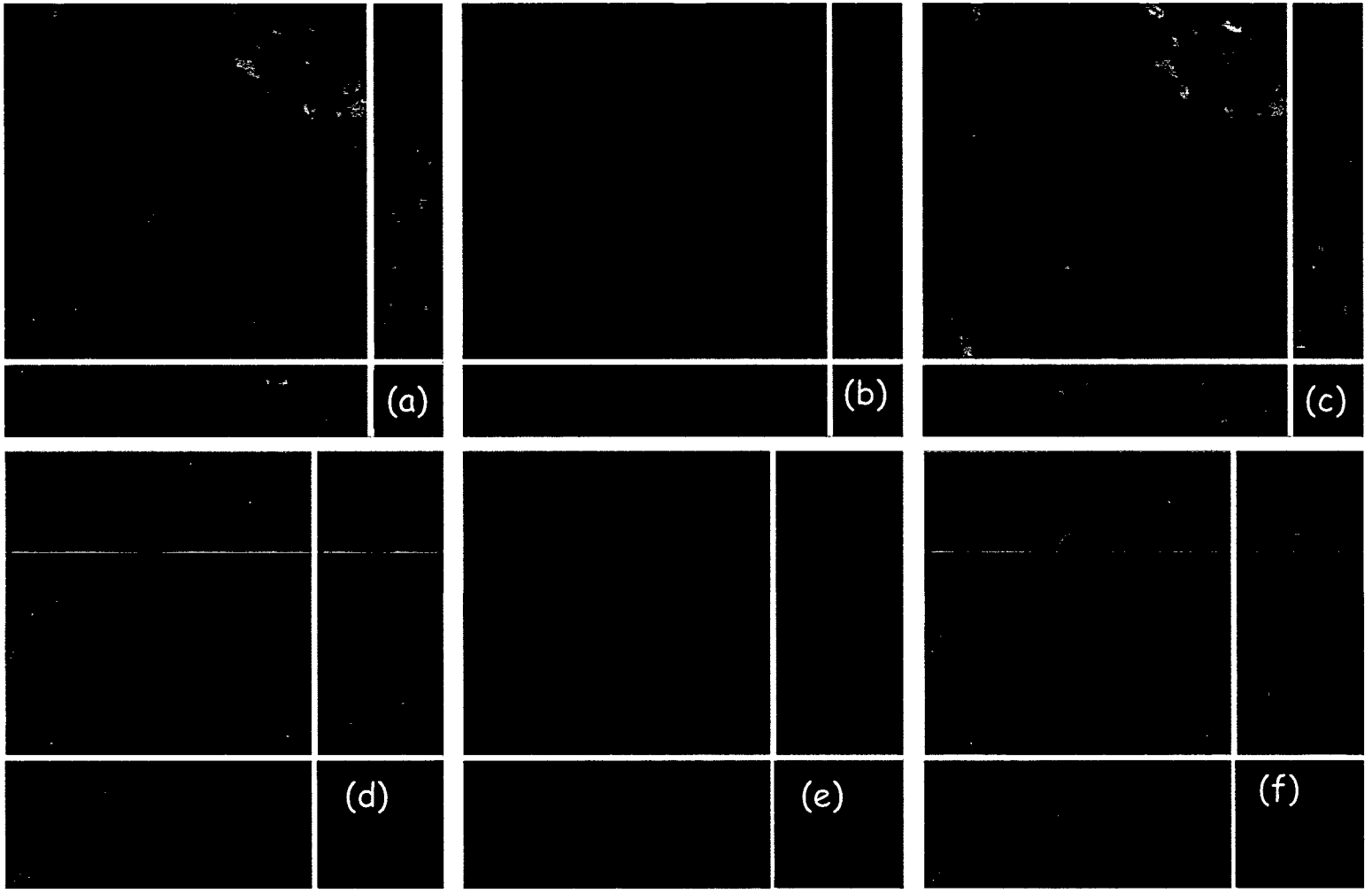

Fig. 12. Identification of proliferating cells within the grafts by double immunostaining for human nuclear marker (HNuc) and BrdU. (a) and (d) Confocal imagse of HNuc positive cells. (b) and (e) Confocal images of BrdU positive cells. (c) and (f) Merged confocal images with double positive cells in yellow.

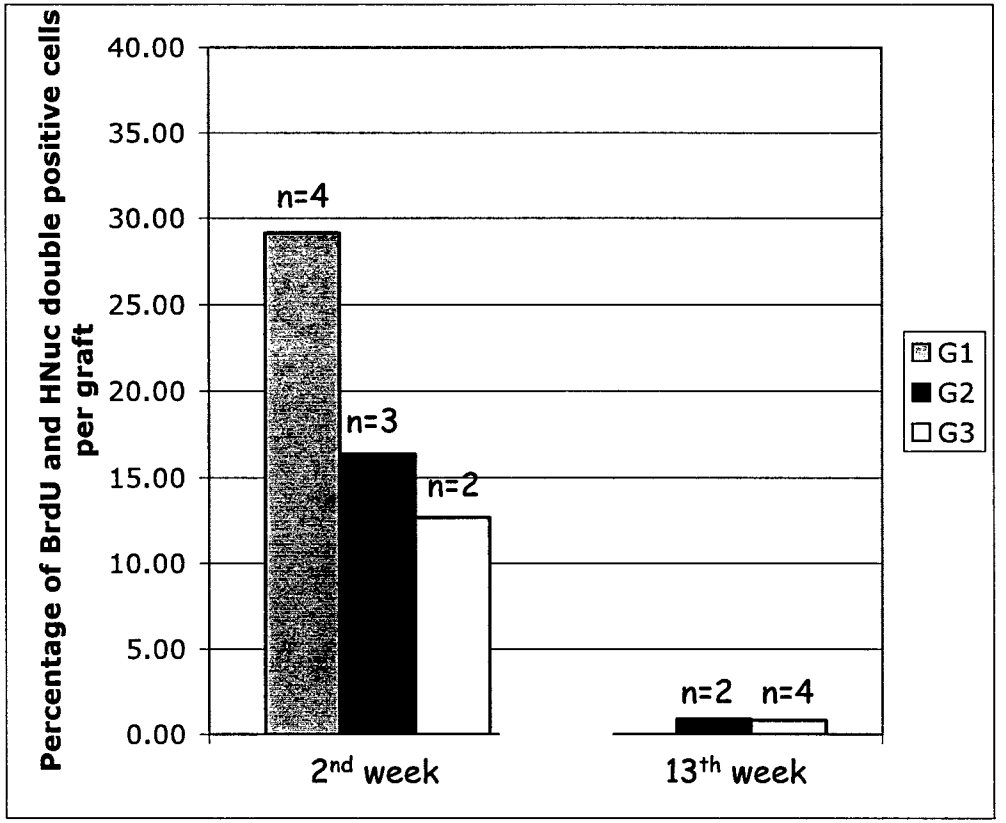

Fig. 13. Stereological analysis of the proliferating cells within the grafts identified by double immunostaining for human nuclear marker (Hnuc) and BrdU. Only brains not showing teratoma formation at the 2nd week and at the 13th week after transplantation are included.

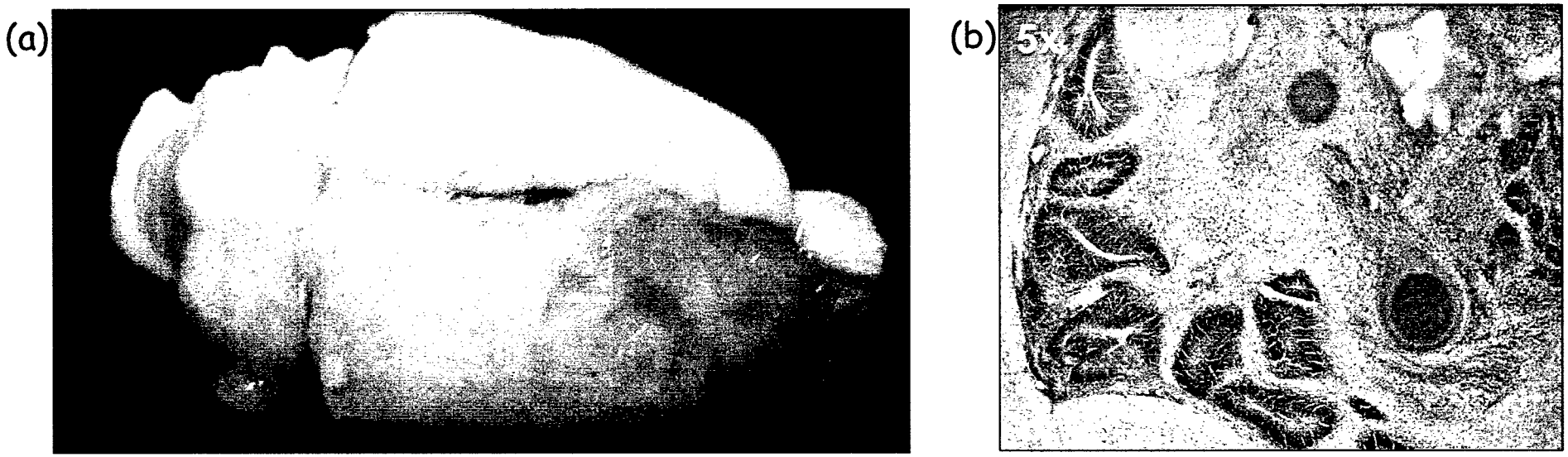

Fig. 14. Teratoma formation. (a) Picture of a brain showing a teratoma in the transplanted site. (b) Image of teratomas in a brain section from a rat of Group 1 showing different tissue structures.

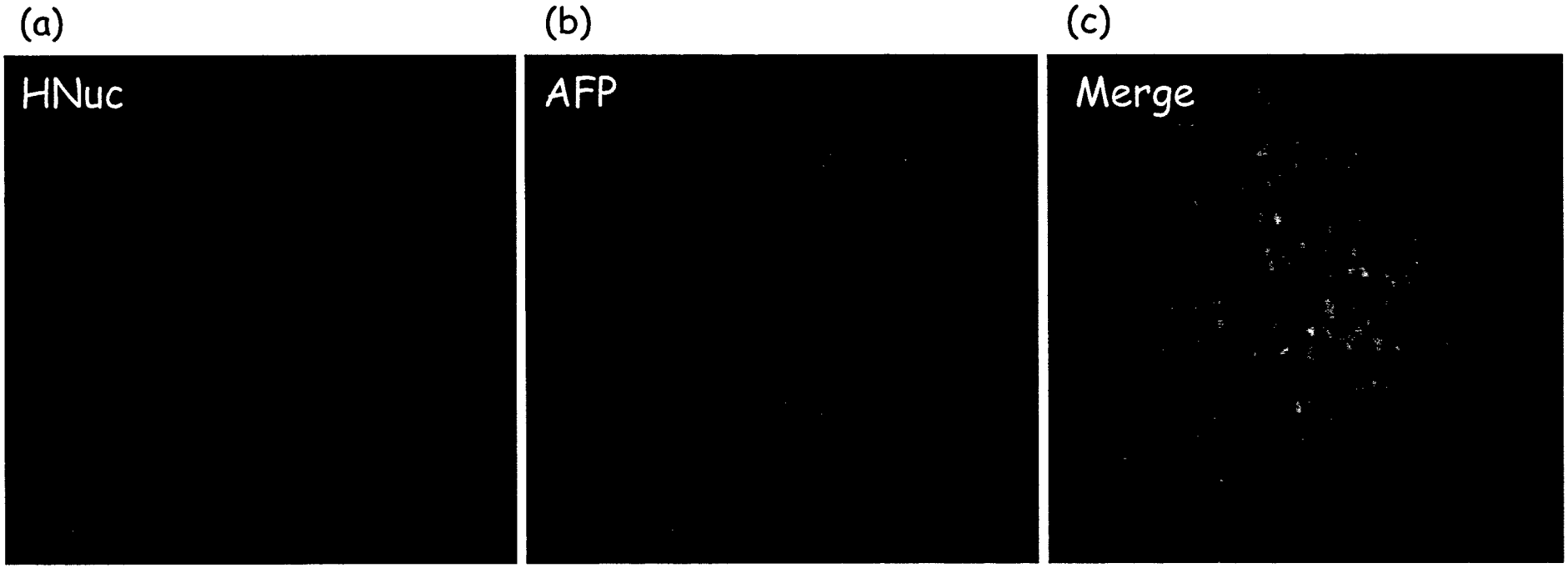

Fig. 15. HESC-derived endodermal cells within the grafts. Endodermal cells were identified by alfa feto protein (AFP) immunostaining. (a) Positive cells for human nuclear marker (HNuc). (b) Positive cells for AFP. (c) Merged picture with double positive cells in yellow.