

Ectopic Dopaminergic Progenitor Cells From *En1*^{+/*Otx2*lacZ} Transgenic Mice Survive and Functionally Reinnervate the Striatum Following Transplantation in a Rat Model of Parkinson's Disease

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Cell-based therapies for Parkinson's disease (PD) using neural stem cells to replace the lost dopamine neurons is currently an intense area of research. In this study we have evaluated the restorative potential of ectopic dopaminergic (DA) neurons derived from the rostral hindbrain (RH) of *En1*^{+/*Otx2*lacZ} transgenic mice. The genetic modification of the DA progenitor domain in the *En1*^{+/*Otx2*lacZ} mice is a gain of function, resulting in the enlargement of the area containing DA neurons, as well as an increase in their absolute number in the midbrain/hindbrain region. Amphetamine-induced rotation performed after cell transplantation into the unilaterally 6-hydroxydopamine-lesioned rat striatum revealed that animals with transgenic RH-derived DA grafts exhibited functional recovery similar to transgenic and wild-type ventral mesencephalon (VM)-derived DA grafts. Morphological analyses revealed equivalent numbers of surviving DA neurons from both homotopic VM- and ectopic RH-derived grafts from transgenic donors with low numbers of surviving serotonergic (5-HT) neurons. Conversely, grafts derived from wild-type donors contained predominantly surviving DA neurons or 5-HT neurons when they were prepared from the VM or RH, respectively. The study demonstrates the pattern of survival and functional potential of ectopic DA neurons derived from the RH of *En1*^{+/*Otx2*lacZ} transgenic mice and that cell transplantation is an important neurobiological tool to characterize newly generated DA neural stem cells in vivo.

Key words: Midbrain/hindbrain boundary; Orthodenticle homolog 2 (*Otx2*); Neuron cell transplantation; DA progenitor domain; Ventral mesencephalon; Nucleus raphe

INTRODUCTION

Dopaminergic (DA) neurons originating from the ventral mesencephalon (VM) play a fundamental role in basal ganglia functions, including governing sensorimotor patterns of behavior, and are primarily affected in Parkinson's disease (PD) (35). A potential therapeutic strategy for PD is the restitution of lost DA neurons by DA-rich grafts derived from the fetal VM or generated in vitro using different protocols and types of stem cells as starting material (11,29,37,38,40). Both preclinical and clinical studies have shown that VM-derived DA cells can innervate the striatum and are able to establish a terminal DA network in the host striatum after trans-

plantation. In contrast, DA neurons derived from other brain regions and investigated so far do not share similar restorative properties (24,41). Further investigations of alternative sources of DA neurons will significantly promote the understanding and development of cell-based restorative approaches for PD (42,45).

Recent studies have demonstrated that the interaction of the transcription factor orthodenticle homolog 2 (*Otx2*) and secreted factor Wnt1 is essential for the early induction and specification of DA neurons (31,32,39) and that this molecular code can be exploited to generate ectopic DA neurons. *En1*^{+/*Otx2*lacZ} transgenic (tg) mice overexpress *Otx2* in the caudal midbrain and rostral hindbrain (RH), leading to a caudal shift of the mid-

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brain/hindbrain boundary (MHB) and, consequently, to an increased number of DA neurons expressing tyrosine hydroxylase (TH) and dopamine transporter (DAT) (7). These DA neurons are distributed rostrally and caudally to the original MHB concomitantly with a reduction of serotonergic (5-HT) neurons in the RH region. It has been previously reported that these ectopically located DA neurons of the RH project to the striatum as a part of the nigrostriatal pathway, and *En1*^{+Otx2lacZ} mice show higher locomotor activity than controls (7). It is unclear, however, if these ectopic but phenotypically DA neurons share the same restorative potential after transplantation as their nigral VM-derived homotopic counterparts. If so, this genetic approach may develop into a valuable strategy for ex vivo cell-based restorative as well as in vivo regenerative therapies.

In this study we analyzed the pattern of TH, 5-HT, and DAT expression in the midbrain/hindbrain region (MHR) of adult transgenic and wild-type (wt) mice, as well as in cell cultures derived from VM and RH of E13.5 mouse embryos. DA-rich cell suspensions were prepared from the RH of E13.5 *En1*^{+Otx2lacZ} mouse embryos and grafted into the ipsilateral striatum of unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats. Survival and functional restorative capacities of RH-derived DA neurons were analyzed and compared to VM-derived grafts, which represent the “gold standard” in this experimental model of PD (11). We provide novel evidence that the ectopic DA neurons derived from the RH exhibit similar morphological and functional capacities as DA neurons derived from the VM and may thus be interesting candidates for further investigations of intrinsic neural regenerative approaches and ex vivo stem cell-based neural repair strategies.

MATERIALS AND METHODS

Experimental Design

Animals were maintained in a constant 12-h light/12-h dark cycle, temperature of $21 \pm 1^\circ\text{C}$, 50–60% relative humidity, and were housed in groups of 4–6 in standard cages with ad libitum access to drinking water and standard food pellets. If not mentioned otherwise, all animals were purchased from Charles River Laboratories (Sulzfeld, Germany). Animal procedures were performed according to guidelines of the German Council on Animal Care and all protocols were approved by the Animal Care Committees of the Universities of Freiburg and Munich.

Mouse embryos (E13.5, crown–rump length: 7–8 mm) were collected from time pregnant wild-type CD1 female mice mated with *En1*^{+Otx2lacZ} transgenic male mice (44). *En1*^{+Otx2lacZ} mice were generated and genotyped as described previously (6). The VM and the RH from

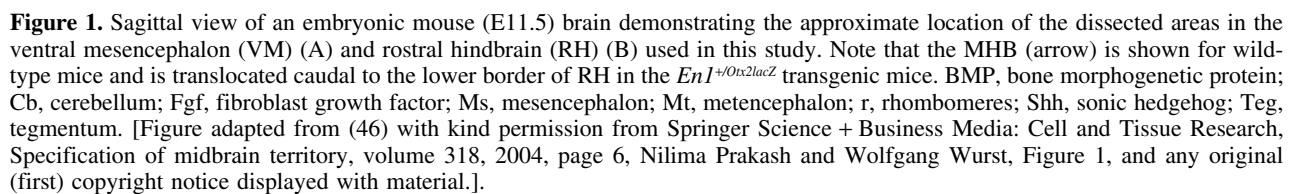
transgenic and wild-type mice embryos were dissected as depicted in Figure 1 and preserved in hibernation medium (Neuromics, USA) for 24 h until genotyping. For the in vitro studies, cells derived from the VM and the RH of wild-type as well as of *En1*^{+Otx2lacZ} transgenic mice were cultured and the number of TH- and 5-HT-expressing cells were analyzed by means of immunocytochemistry.

For the in vivo transplantation studies, adult female Sprague-Dawley rats weighing 250–300 g received a unilateral 6-OHDA medial forebrain bundle (MFB) lesion. All animals were operated under deep anesthesia induced by IP injection of ketamine (80 mg/kg; Ceva, Germany)/xylazine (4 mg/kg; Rompun Bayer, Germany).

Lesion effects were evaluated by apomorphine- and amphetamine-induced rotations 6 weeks after lesion. Only rats exhibiting a mean net rotation of at least four full turns/min for apomorphine and six turns/min for amphetamine over 40 min and 90 min, respectively, were included in the study. This criterion ensured that the 6-OHDA-induced DA denervation of the caudate-putamen unit was complete (17). Successfully lesioned animals were matched into five groups based on their rotation data. Rats were transplanted 7 weeks after the lesion with the mouse embryo E13.5 tissue (Fig. 1) in the four different experimental groups from two regions and two genetic backgrounds: i) wild-type ventral mesencephalon (wtVM, $n = 9$); ii) wild-type rostral hindbrain (wtRH, $n = 10$); iii) transgenic ventral mesencephalon (tgVM, $n = 10$); and iv) transgenic rostral hindbrain (tgRH, $n = 10$). A lesioned and sham-transplanted group (sham, $n = 10$) and a control group with healthy animals ($n = 7$) were also included. Transplantation effects were evaluated by drug-induced rotations 2 and 5 weeks after transplantation. All animals were sacrificed 7 weeks after transplantation and the grafts were evaluated by morphological analysis. The numbers of TH-, 5-HT-, and DAT-expressing cells in the VM and the RH of wild-type and transgenic *En1*^{+Otx2lacZ} (6) mice were quantified by means of stereology.

Unilateral 6-OHDA MFB Lesion

Adult rats received a unilateral lesion of the nigrostriatal DA pathway by two stereotactic injections of 6-OHDA (Sigma, Germany) into the right medial forebrain bundle (MFB), using 10- μl Hamilton syringes with a 26-gauge steel cannula. The following coordinates were used (in mm with reference to bregma and dura): first tract anteroposterior (AP) -4.0 , lateral (L) -0.8 , ventral (V) -8.0 ; tooth bar (TB) $+3.4$; second tract: AP -4.4 , L -1.2 , V -7.8 ; TB -2.3 . Every animal received a total amount of 5.5 μl of 1.75×10^{-2} M 6-OHDA solution (first track: 3.0 μl , second track: 2.5 μl) with an



Seven weeks after the 6-OHDA lesion, single cell suspensions from all areas of interest (wtVM, wtRH,

tgVM, tgRH) of E13.5 embryos were prepared according to a protocol (27–30) based on the standard cell suspension technique of Björklund and colleagues (4). Briefly, the tissue was incubated in 0.1% trypsin (Worthington, USA), 0.05% DNase (SigmaDN-25; Sigma, Germany), and DMEM (Gibco, Germany) at 37°C for

20 min, rinsed four times in 0.05% DNase/DMEM, and mechanically dissociated by trituration through a 1-ml and a 200- μ l pipette. The tissue was then centrifuged at 600 rpm for 5 min, and the pellet was resuspended in 0.05% DNase/DMEM. The cell number of each cell suspension was adjusted to 110,000–120,000 cells/ μ l. The viability was >95% before transplantation as determined by trypan blue dye exclusion assay. Micrografts from each cell suspension were implanted using a glass capillary (outer diameter = 50–70 μ m) connected to a 2- μ l Hamilton microsyringe (27,28). Two deposits of 1 μ l each were placed along each of two implantation tracts in the head of the caudate-putamen, resulting in a total of four micrografts with a total graft volume of 4 μ l per animal at the following coordinates (in mm, with reference to bregma and dura): AP +1.0; L $-2.5/-3.3$; V $-5.0/-4.0$; TB 0. Starting 1 day before and daily after the transplantation, all transplanted animals were immunosuppressed by IP injections of cyclosporine (10 mg/kg body weight/day; Sandimmun®; Novartis, Switzerland).

Drug-Induced Rotations

Drug-induced rotations were performed as previously described (43). Six weeks after the 6-OHDA lesion surgery and at 2 and 5 weeks after transplantation, the animals were given SC injections of apomorphine (0.05 mg/kg body weight, Sigma, Germany) in 2 mg/ml ascorbic acid/saline. The rotational behavior was monitored over a period of 40 min in automated rotometers. Two days later, 2.5 mg/kg body weight of D-amphetamine (Sigma, Germany) diluted in saline was given IP, and the rotational bias was observed over a period of 90 min.

Cell Culture

In parallel with the single cell suspension prepared for the transplantation, cell suspensions were prepared from the VM and the RH of E13.5 wild-type and transgenic *En1^{+0m2lacZ}* mouse embryos (groups wtVM, wtRH, tgVM, tgRH). The preparation was identical as in the *in vivo* studies. Cells were plated at a density of 100,000 cells/cm² on 0.01% poly-L-ornithine (Sigma, Germany) precoated 48-well plates and were cultured overnight (O/N) in Neurobasal (Gibco, Germany) medium supplemented with 10% fetal calf serum (FCS; Sigma, Germany), 2% B27 (Gibco, Germany), 1% Glutamax (Gibco, Germany), 1% penicillin-streptomycin-ampicillin (Gibco, Germany). After O/N incubation the medium was replaced with the same one devoid of FCS and thereafter changed every 2 days. Cells were cultured at 37°C in 5% CO₂ and 21% O₂ for 1 and 4 days before fixation with 4% ice-cold PFA for 45 min. Fixed cells were washed three times with PBS, permeabilized with 0.1% Triton X-100 (Sigma, Germany) diluted in PBS, and blocked for unspecific antibody binding with 4% goat serum (Sigma, Germany) at room temperature (RT)

for 2 h. Cells were then incubated O/N at 4°C with the following primary antibodies: β -III-tubulin (1:300; Covance, USA), TH (1:2,500; Sigma, Germany), β -III-tubulin (1:500; Chemicon, Germany), activated caspase-3 (1:300; BD Pharmingen, USA), 5-HT (1:250; MBBiochem, Germany). The next day cells were washed three times with PBS and incubated at RT for 2 h with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (1:10,000; Sigma, Germany) and secondary Alexa antibodies (Alexa Fluor®; Invitrogen, Germany). Cells were washed three times with PBS, 0.001% Tween (Calbiochem, Germany) in PBS, and H₂O and stored in 0.05% acetic acid/PBS at 4°C. All *in vitro* experiments were performed in duplicate and repeated twice.

Immunohistochemistry

Animals received terminal IP doses of ketamine (Ceva, Germany)/xylazine (Bayer, Germany) and were transcardially perfused with 0.1 M PBS, followed by ice-cold 4% paraformaldehyde (PFA) (Merck, Germany), pH 7.2. The brains were postfixed for 2–12 h in PFA and dehydrated for 24–48 h in 30% sucrose (Calbiochem, Germany)/PBS. Serial coronal sections (40 μ m thick) were cut on a freezing microtome. Every fourth section was processed for TH, 5-HT, and DAT immunohistochemistry as follows. Free-floating sections were washed three times with PBS, quenched with 3% H₂O₂/10% methanol/PBS for 10 min, and washed three times with PBS. After 2-h preincubation in 3% bovine serum albumine (BSA; Sigma, Germany)/0.3% Triton X-100 (Sigma, Germany)/PBS, the sections were incubated overnight at RT with the primary antibodies TH (1:2500; Sigma, Germany), 5-HT (mouse-specific antibody does not label rat derived 5-HT) (1:250; MBBiochem, Germany), or DAT (1:250, Chemicon, Germany) in 3% BSA/0.3% Triton X-100/PBS. After three washes with PBS, sections were incubated for 2 h with a biotinylated secondary antibody (1:200; Dako, Germany) or with an Alexa secondary antibody (Alexa Fluor® 1:200; Invitrogen, Germany) in 3% BSA/0.3% Triton X-100/PBS. Visualization for the biotinylated antibody was carried out with the ABC-kit (Vector, Germany) and DAB (Merck, Germany). DAB-stained sections were mounted on glycerin-coated glass slides, dehydrated in ascending alcohol concentrations (70%/95%/100%; 10 min each), and cover slipped with Histofluid mounting medium (Zitt-Thoma, Germany). Fluorescence-stained sections were cover slipped with fluorescent mounting medium (Dako, Germany).

Cell Counting

The numbers of DAPI⁺, β -III-tubulin⁺, TH⁺, and 5-HT⁺ cells were quantified by fluorescent microscopy analysis; 100–200 DAPI⁺ nuclei were counted in 1/20

of a grid and β -III-tubulin⁺ cells in 1/5 of a grid per well in four wells for each treatment and/or cell type. TH⁺ or 5-HT⁺ cells were counted in the same grids as DAPI and β -III-tubulin. The total cell number based on DAPI⁺ staining as well as the total cell number of β -III-tubulin⁺ and TH⁺ cells per well were calculated. β -III-tubulin⁺, TH⁺, and 5-HT⁺ cell numbers are expressed as percentages in relation to the total cell number based on DAPI⁺ cells.

Stereological Analysis

The number of 5-HT⁺ cells in the RH as well as TH⁺ cells in the VM of wild-type ($n = 6$) and transgenic ($n = 6$) *En1^{+/Otx2lacZ}* mice (including the additional caudal population) and the ventral tegmental area (VTA) were quantified using the optical fractionator method (Stereoinvestigator system, MicroBrightField Bioscience, Germany) (14,22). The coexpression of TH and DAT was determined in TH⁺ cells. TH- and 5-HT-immunoreactive graft-derived neurons in the striatum were counted under bright field illumination and 10 $\times$ magnification. An approximation of the total graft cell number was calculated according to the formula of Abercrombie (1). Graft volumes were determined according to the Cavalieri method (14,22) with the help of a computerized stereology system (Stereoinvestigator; MicroBrightField Bioscience, Germany).

Statistical Analysis

All statistical analyses were performed with the software StatView (Abacus, UK) or GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, USA) using a one-way or two-way ANOVA followed by the Student-Newman-Keuls post hoc test. Level of significance was set at $p < 0.05$. Results are expressed as means $\pm$ SEM.

RESULTS

TH⁺ and 5-HT⁺ Cell Populations Rostral and Caudal to MHB

The increase in TH⁺ volumes and cell numbers in the VM and RH of adult *En1^{+/Otx2lacZ}* mice reported by Brodski et al. (7) could have been due to a reduced density of these cells in the transgenic brain, an issue that was not addressed in the previous study. Therefore, we reassessed the number of the TH⁺ and 5-HT⁺ cells in the area rostral and caudal to the MHB of wild-type and transgenic animals, using a well-established and unbiased stereological method. Cell counts in the VM (Fig. 2) revealed a small but nonsignificant increase in the total number of TH⁺ cells in transgenic (8104 ± 807 ; $n = 6$) compared to wild-type (7052 ± 369 ; $n = 6$) brains. However, the area covered by TH⁺ cells (Fig. 3) was significantly larger in transgenic mice ($1038 \times 10^6 \pm 94 \times 10^6 \mu\text{m}^3$) compared to wild-type animals

[$558 \times 10^6 \pm 69 \times 10^6 \mu\text{m}^3$; $F(1, 10) = 17.1$, $p = 0.002$]. TH⁺ cells could be observed until -5.00 mm posterior in transgenic mice whereas the wild-type mice displayed only TH⁺ cells until -4.00 mm posterior (all coordinates are given with reference to the bregma). In both groups TH⁺ cells coexpressed DAT to the same extent (transgenic $78.8 \pm 1.6\%$; wild-type $79.3 \pm 2.1\%$; % of DAT⁺ in the TH⁺ cell population) (Fig. 4). Furthermore, transgenic mice had significantly lower 5-HT cell numbers in the RH [699 ± 52 cells; $F(1, 10) = 5.4$, $p = 0.0431$] (Figs. 2 and 5) distributed over the same volume ($122.1 \times 10^6 \pm 13.8 \times 10^6 \mu\text{m}^3$) (Fig. 3) compared to wild-type mice [909 ± 74 cells, in $116.2 \times 10^6 \pm 17.0 \times 10^6 \mu\text{m}^3$; $F > (1, 10) = 0.07$, $p = 0.79$]. The regions containing 5-HT⁺ cells ranged from AP -4.00 mm to -8.10 mm in transgenic, and from AP -3.90 mm to -8.10 mm in wild-type mice: the TH⁺ and 5-HT⁺ domains overlapped much more in the transgenic animals, while a predominantly 5-HT presence was noted in the RH in the wild-type animals (Fig. 5).

Cell Cultures of Transgenic- and Wild-Type-Derived MHB Regions

Because transgenic adult *En1^{+/Otx2lacZ}* mice have an ectopic functional DA population, we wanted to study the behavior of these cells under in vitro cell culture conditions. Cultures derived from wtVM, tgVM, wtRH, and tgRH of *En1^{+/Otx2lacZ}* E13.5 mouse embryos (Fig. 6) were analyzed under phase microscopic illumination and they showed a comparable growth of cells in all four groups.

After 1 day of in vitro (1 DIV) culture, the percentage of β -III-tubulin⁺ neuronal cells (Fig. 6A) was significantly higher (+8–15%) in the wtVM cells than in the three other groups [$F(3, 28) = 10.1$, $p < 0.05$]. This difference was not found after 4 DIV culture (Fig. 6B). Analysis of 5-HT⁺ cells (Fig. 6C, D) revealed significantly higher percentages in wtRH-derived cultures (1.7%) than in all other groups (0.3–0.45%); this group also showed significantly lower percentage of DA cells (0.2%) (Fig. 6E, F). These results were independent from culture time [1 DIV: $F(3, 12) = 68.7$, $p < 0.0001$; 4 DIV: $F(3, 12) = 354.9$, $p < 0.0001$].

There were significantly higher proportions of TH⁺ cells (Fig. 6E) in cultures derived from wtVM (2.4%) than in the other groups following 1 DIV [0.2–1.45%; $F(3, 12) = 50.5$, $p < 0.0004$]. Cultures prepared from tgVM tissue contained 1.45% of TH⁺ cells compared to 0.45% TH⁺ cells derived from the RH [$F(3, 12) = 50.5$, $p < 0.001$] (Fig. 6E). After 4 DIV the analysis of TH⁺ cell counts (Fig. 6F) did not show a significant difference any longer between transgenic and wild-type VM. However, there was still a significant difference between tgVM-derived (0.97%) and tgRH-derived (0.35%) cultures [$F(3, 12) = 34.3$, $p = 0.0002$].

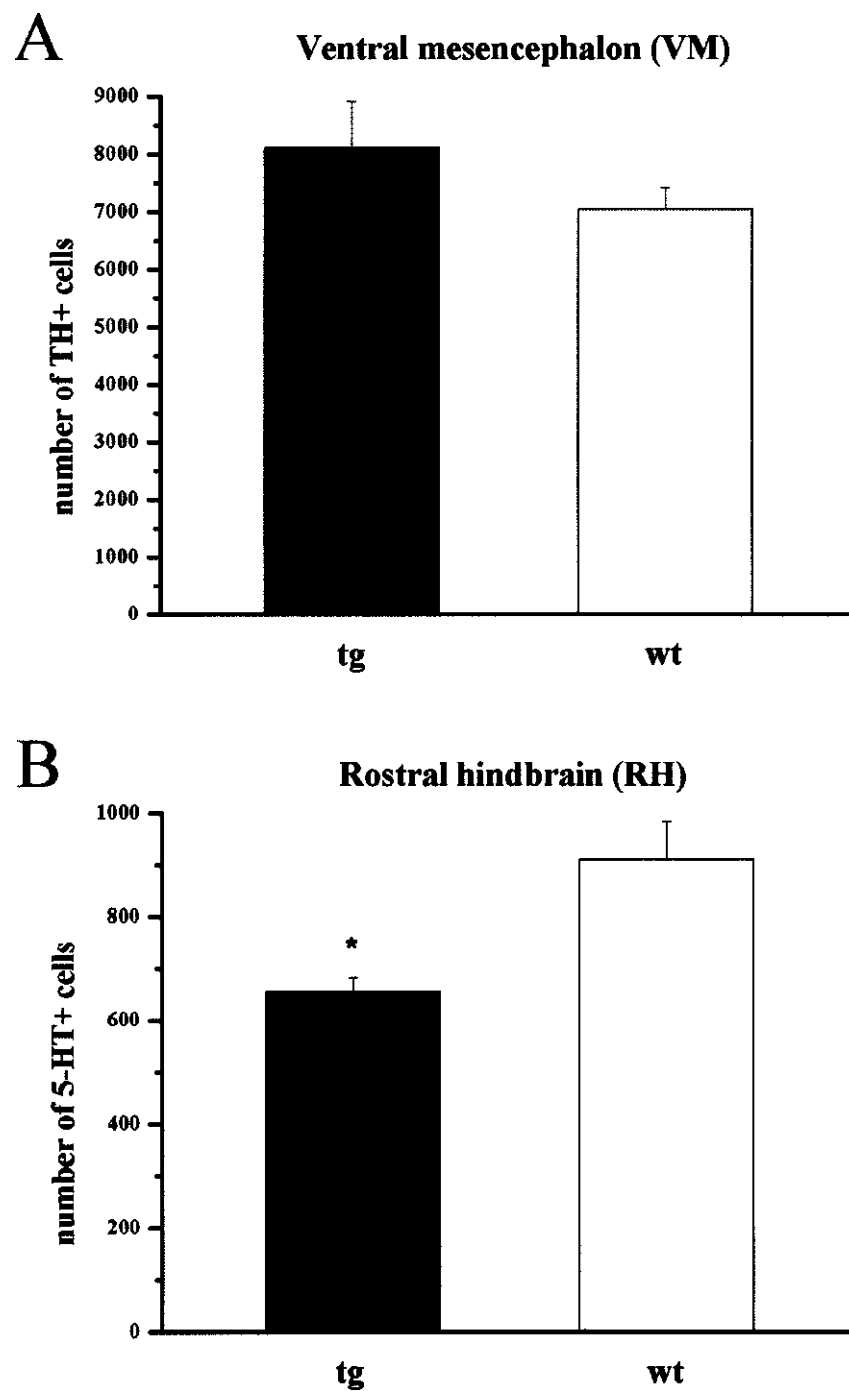


Figure 2. The total number of TH⁺ neurons in the VM (A) and 5-HT⁺ neurons in the RH (B) in adult wild-type and transgenic *En1^{+/Ox2lacZ}* mice. There were 15% more TH⁺ neurons in the VM in transgenic mice compared to wild-type mice (8104 ± 807 vs. 7052 ± 369), although this did not reach statistical significance. Wild-type mice had significant larger numbers of 5-HT neurons than transgenic mice (+30%, 909 ± 74 vs. 699 ± 52; $p < 0.05$). *Significant different to the wild-type mice.

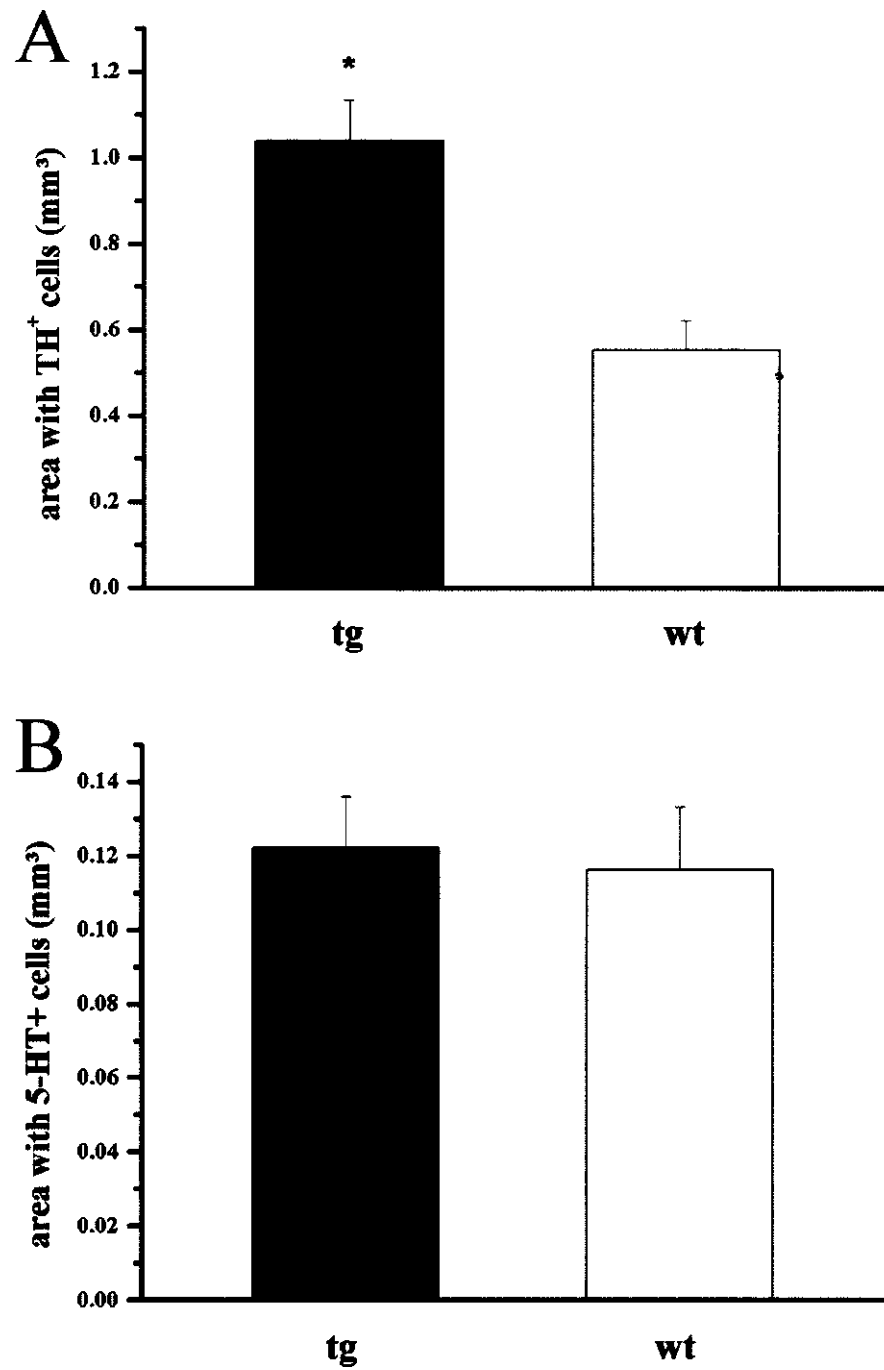


Figure 3. The area in the m/h region over which TH⁺ (A) and 5-HT⁺ (B) were distributed in adult wild-type and transgenic *En1*^{+/Otx2lacZ} mice. Interestingly, there was a significant larger area covered by TH⁺ neurons in transgenic than in wild-type mice (+86%, 1.04 ± 0.01 mm³ vs. 0.56 ± 0.07 mm³, $p < 0.01$). In contrast, the area of 5-HT⁺ neurons was not significantly different between both groups.

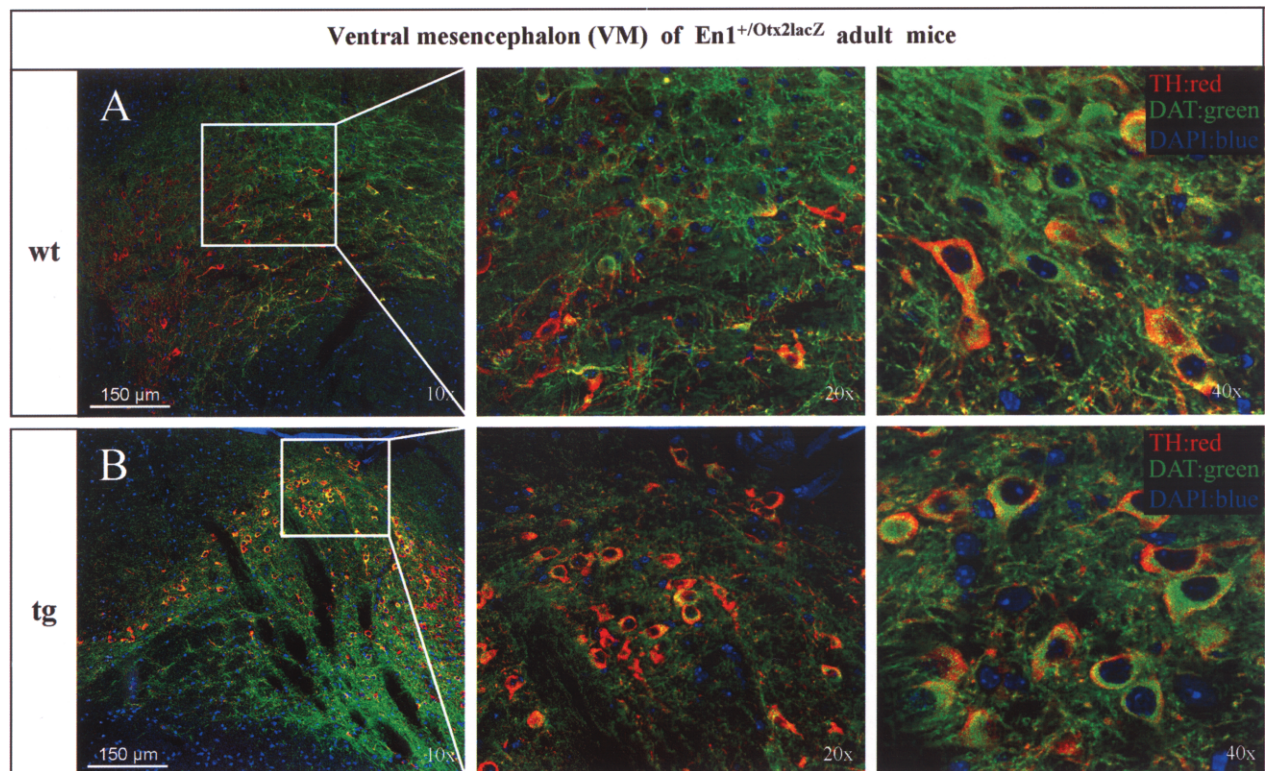


Figure 4. TH (red) and DAT (green) expression in the VM in adult wild-type (A) and transgenic (B) *En1*^{+/Otx2lacZ} mice. Nuclei staining was performed with DAPI (blue). There was a high coexpression of TH and DAT (~80%) in VM neurons in both transgenic and wild-type mice with a similar morphological appearance. However, the density of TH⁺ neurons in transgenic mice was higher within the whole VM area compared to wild-type mice.

We also performed activated caspase-3 immunostaining in order to compare apoptotic rates across the different cell cultures. Cells fixed after 1 DIV did not show any significant differences between the groups. The percentages of cells being positive for activated caspase-3 ranged between 0.62% and 1.4% of the total DAPI⁺ cell number (data not shown).

Graft-Induced Functional Behavioral Effects

In order to explore the functional potential of the transgenic DA neurons, cell suspension grafts from the VM and RH both from transgenic and wild-type embryonic donors were implanted unilaterally into 6-OHDA-lesioned rats and, as a functional measure, drug-induced rotational behavior was tested. Figure 7A demonstrates the apomorphine-induced, and Figure 7B the amphetamine-induced rotational behavior. The rotational bias postlesion and 2 and 5 weeks after transplantation is shown for every experimental group. No significant differences were observed between the four experimental groups 5 weeks postlesion, with all animals showing severe asymmetrical rotational behavior following both apomorphine and amphetamine administration, with the control animals not rotating at all.

Two weeks postgrafting both groups containing VM cells (tgVM and wtVM) showed a nonsignificant tendency of a reduced apomorphine-induced rotational asymmetry. Five weeks postgrafting, however, both VM groups (wild-type as well as transgenic) showed a significant recovery of about 54% compared to the sham-transplanted animals [postlesion vs. 5 weeks: wtVM $F(2, 24) = 5.8$, $p < 0.05$; tgVM: $F(2, 27) = 4.7$, $p < 0.05$; wtVM/tgVM vs. sham $F(2, 150) = 26.6$, $p < 0.01$]. A statistical trend of reduced rotational bias after apomorphine-induced rotation was observed in the tgRH rats [tgRH postlesion vs. 5 weeks: $F(2, 27) = 1.5$, $p = 0.09$, n.s.]. Following amphetamine administration 2 weeks postgrafting, both groups containing VM tissue showed a significant reduction in their rotational asymmetry compared to their postlesion performance as well as compared to sham-operated animals [wtVM: -1.9 ± 2.4 tpm, postlesion vs. 2 weeks $F(2, 24) = 10.93$, $p < 0.05$; tgVM: 0.1 ± 2.1 tpm, postlesion vs. 2 weeks $F(2, 27) = 14.5$, $p < 0.05$; wtVM/tgVM vs. sham: $F(2, 150) = 26.6$, $p < 0.01$]. Importantly, the animals that had received tgRH cells also showed a significant reduction of their rotational behavior [tgRH: 0.7 ± 1.9 tpm, postlesion vs. 2 weeks $F(2, 27) = 14.3$, $p < 0.05$]. In contrast, the

wtRH group remained stable at a rotation of 11.3 ± 1.3 tpm and was not significantly different from the group of sham-transplanted animals. The results 5 weeks after grafting show that the DA graft-induced improvement in rotational behavior observed in the groups tgVM, wtVM, and tgRH remained stable compared to the results 2 weeks after the transplantation [postlesion vs. 2 weeks: wtVM $F(2, 24) = 10.9$, $p < 0.05$; tgVM $F(2, 27) = 14.5$, $p < 0.05$; tgRH $F(2, 27) = 14.3$, $p < 0.05$].

Survival of TH⁺ Neurons In Vivo

Following the functional assessment, the pattern of graft survival and neuron cell differentiation was evaluated by immunocytochemical means. Immunohistochemical staining against TH for all transplanted groups is presented in Figure 8. All animals were unilaterally lesioned and transplanted into the ipsilateral hemisphere,

with the DA striatal innervation in the contralateral hemisphere remaining intact. Both VM-grafted groups (tgVM and wtVM) displayed good TH⁺ cell survival within the transplanted striatum, but the wtRH grafts contained very few TH⁺ cells. In contrast, animals receiving the equivalent tissue from the transgenic donors (tgRH) exhibited both good survival and integration of TH⁺ cells within the host striatum. Figure 9A illustrates the proportion of surviving intrastriatal TH⁺ neurons in the grafts. No TH⁺ neurons were found in control or sham animals. In the wtVM group 1400 ± 165 TH⁺ neurons were counted, which was a significantly higher survival rate than observed in all other groups [$F(3, 35) = 21.6$, $p < 0.0001$]. In contrast, wtRH grafts contained 94 ± 16 TH⁺ cells, which was not significantly different to control and sham animals [$F(5, 50) = 33.5$, $p > 0.05$]. Both groups transplanted with cells from the transgenic

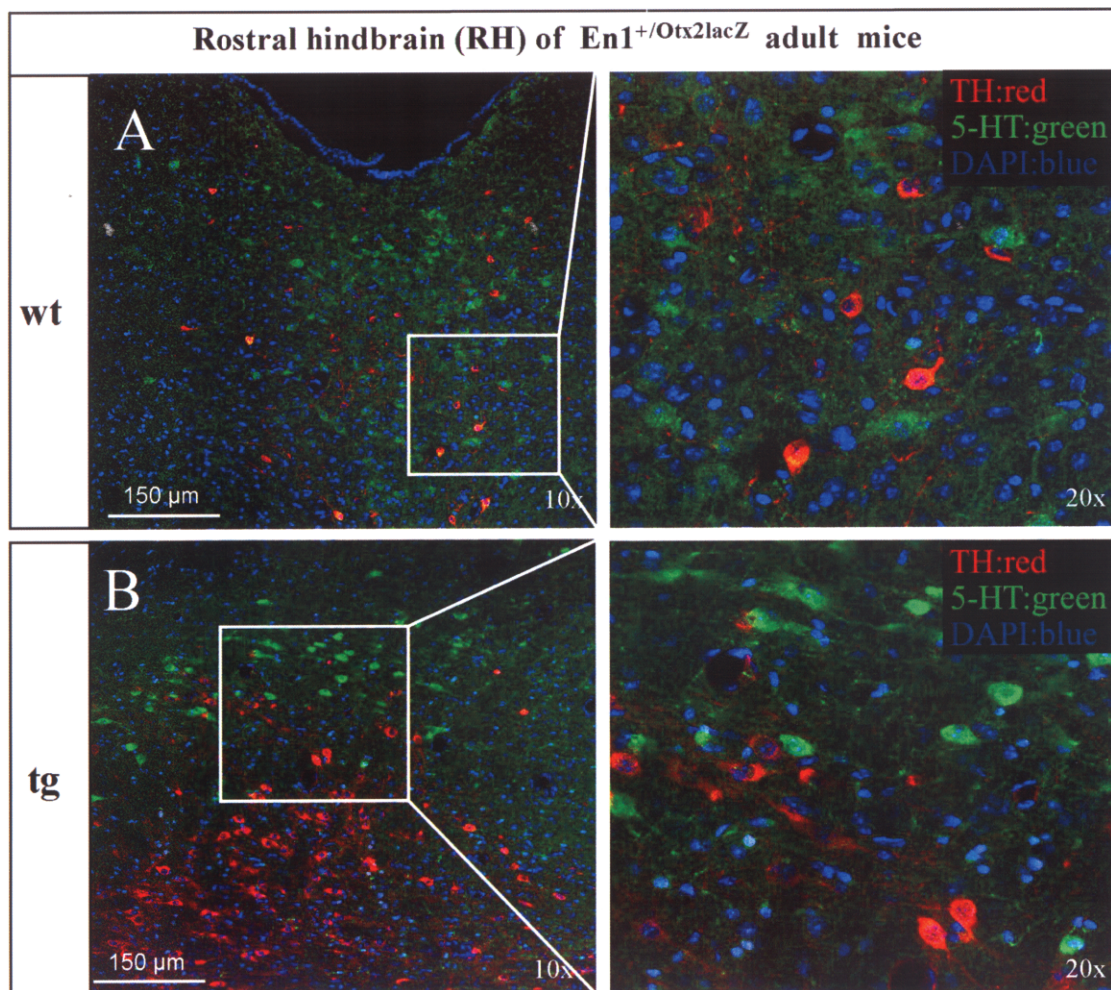


Figure 5. TH (red) and 5-HT (green) expression in the RH region in adult wild-type (A) and transgenic (B) *En1*^{+/Otx2lacZ} mice. Nuclei staining with DAPI (blue). Note that the TH and 5-HT expression domains appear overlapping in transgenic mice, whereas a predominant 5-HT expression was found in the RH in wild-type animals. The inset panels were further magnified from 10× to 20×.

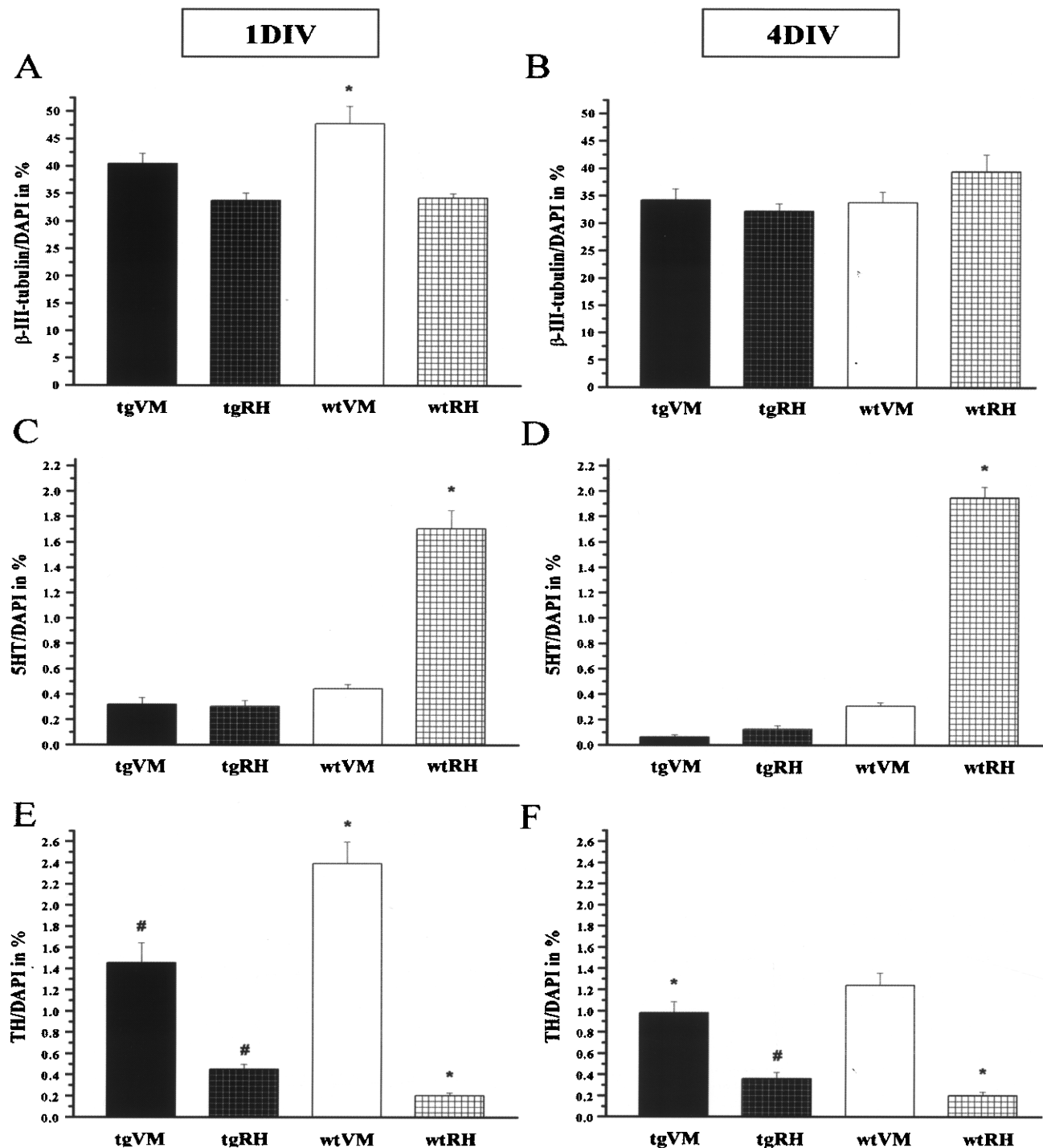


Figure 6. Evaluation of β -III-tubulin (A, B), 5-HT (C, D), and TH expression (E, F) levels of VM- and RH-derived cell cultures from transgenic and wild-type E13.5 *En1*^{+/Otx2lacZ} mouse embryos after 1 and 4 days in vitro (DIV). There was a trend for increased β -III-tubulin expression in the VM compared to the RH, which reached significance in wild-type on 1 DIV (+15%, $p < 0.05$), but was not seen on 4 DIV. Further quantification revealed a significantly higher percentage of 5-HT-expressing neurons in wtRH-derived cell cultures ($1.7 \pm 0.12\%$) compared to all other regions tested (between 0.3% and 0.45%) on 1 DIV and on 4 DIV ($p < 0.001$). The levels of TH⁺ neurons were highest in wtVM-derived cell cultures ($2.4 \pm 0.21\%$) and significantly lower in tgVM ($1.45 \pm 0.18\%$), tgRH ($0.45 \pm 0.05\%$), and wtRH ($0.2 \pm 0.03\%$) cultures on 1 DIV. Comparable findings, although on an overall lower level, were made on 4 DIV. *Significant differences against all other cultures; #significant differences against the other transgenic region.

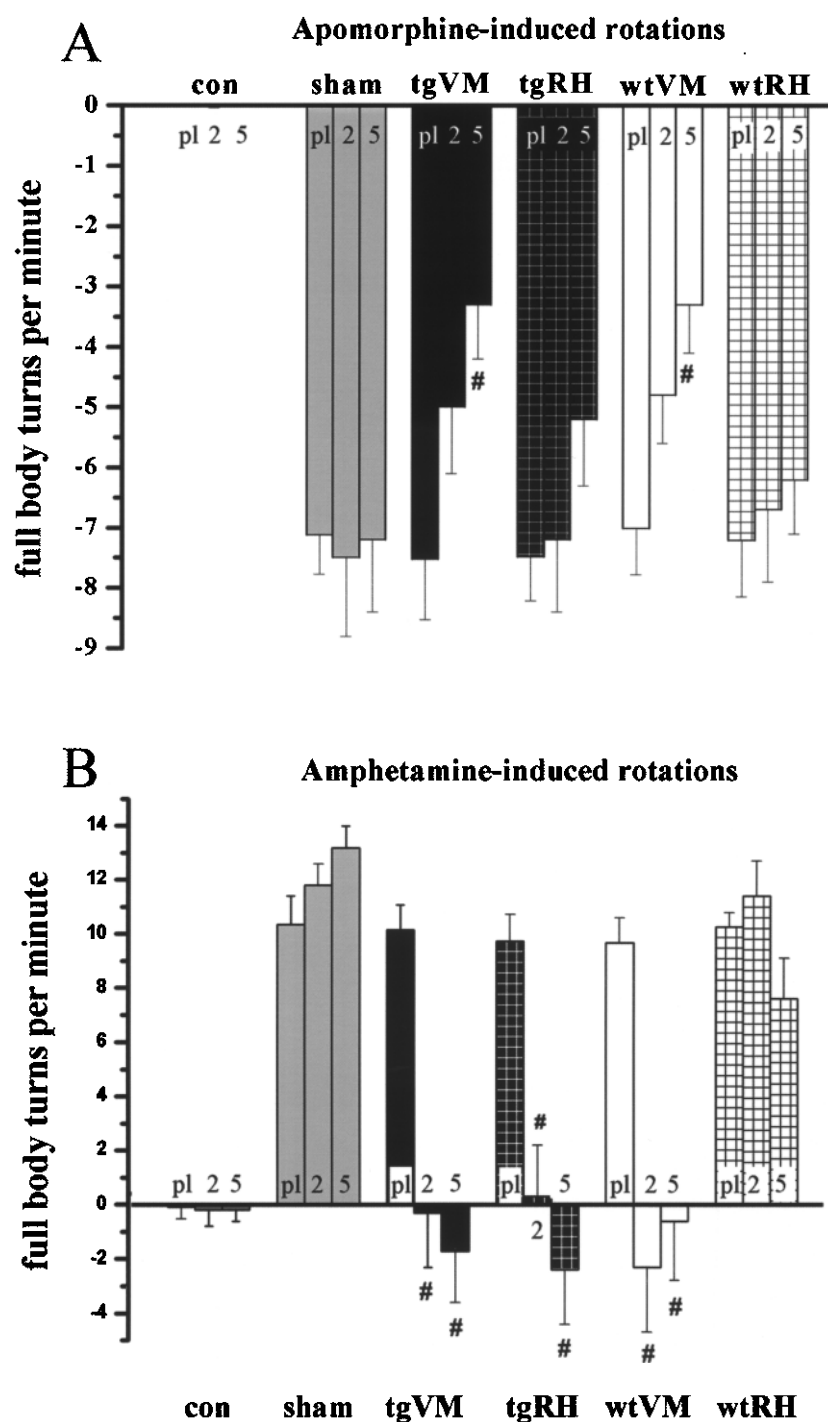


Figure 7. Drug-induced rotation tests. Following the administration of either apomorphine (A) or amphetamine (B) the rotational behavior was monitored postlesion (pl), 2 and 5 weeks postgrafting. (A) Apomorphine-induced rotation displayed -7.5 tpm for all groups except the control nonlesioned animals 2 weeks after the lesion. The rotational asymmetry was partly reduced for the tgVM and wtVM groups 2 weeks after grafting and significantly reduced 5 weeks after grafting (-3.4 ± 0.9 tpm, $p < 0.05$). The tgRH group showed a nonsignificant trend towards reduced rotational scores; no effect was seen in wtRH animals. (B) Amphetamine-induced rotation reached 10 tpm postlesion and was significantly reduced 2 weeks after grafting in the tgVM, tgRH, and wtVM graft groups. Those latter three groups fully compensated or overcompensated their rotational asymmetry scores 5 weeks after transplantation ($p < 0.01$). In contrast, wtRH animals showed no changes in their rotational response 5 weeks after grafting. #Significant differences from postlesion scores and sham-transplanted animals.

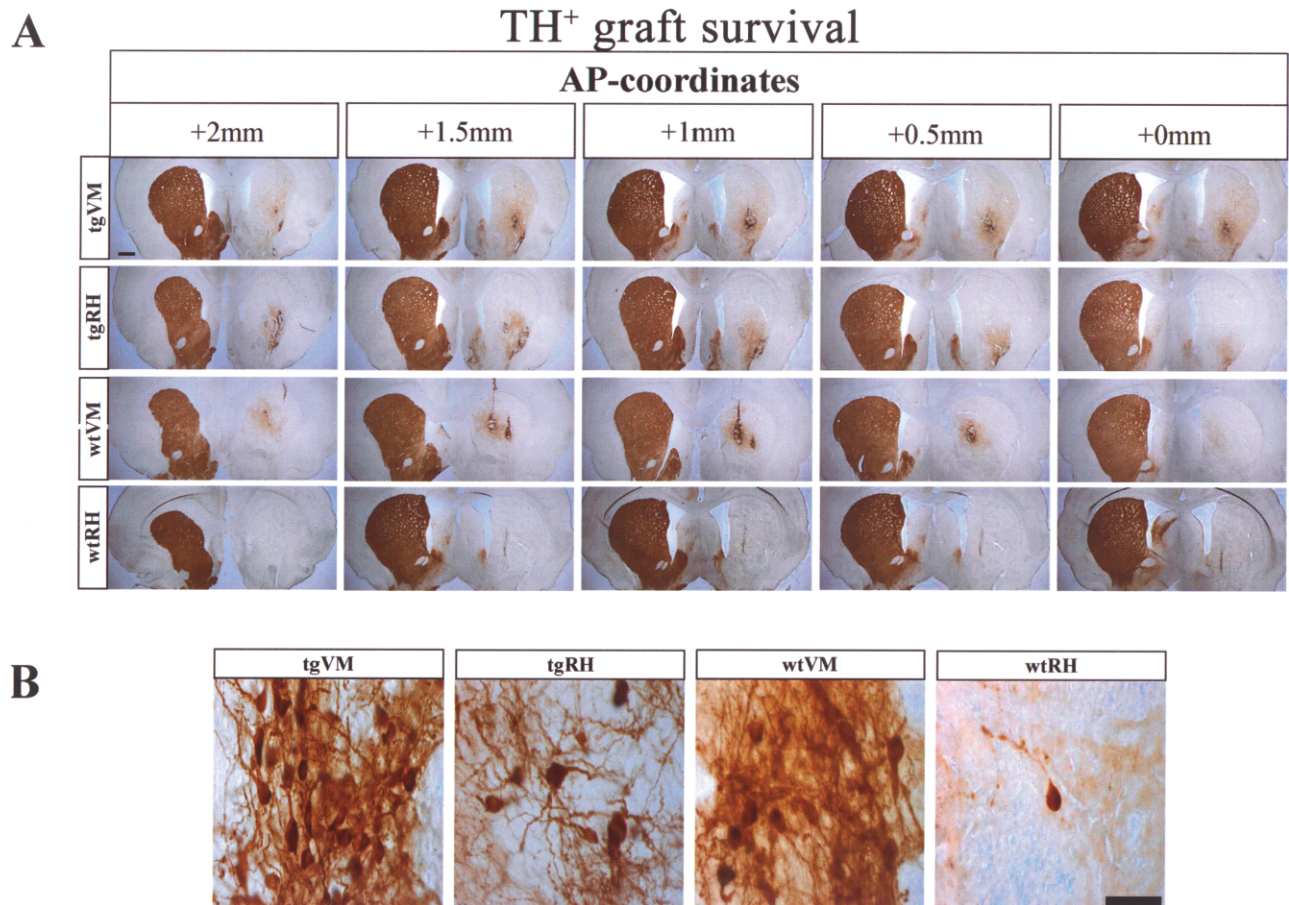


Figure 8. TH⁺ immunohistochemistry on coronal sections illustrating TH⁺ cell survival and striatal reinnervation on the lesioned and grafted ipsilateral striatum (right-hand side). Data were compared to the intact contralateral striatum (left-hand side) for all experimental groups. The groups receiving DA-rich grafts (tgVM, tgRH, and wtVM) exhibited good TH⁺ neuron survival and moderate TH⁺ fiber outgrowth into the host striatal neuropil (A). In contrast, wtRH-derived grafts showed very few surviving TH⁺ neurons and no TH⁺ fiber outgrowth in the host striatum, as shown in representative high magnification images (B). Anterior posterior (AP)-coordinates are given in relation to bregma. Scale bar: 1 mm (A) and 30 μ m (B).

donors showed an average TH⁺ cell survival that was significantly different from both wild-type groups [tgVM 846 ± 119 cells, tgRH 713 ± 120 cells; $F(3, 35) = 21.6$, $p < 0.0001$]. Furthermore, the wtVM group had a significantly larger graft volume (0.18 ± 0.08 mm³) compared to all other experimental groups [0.025 mm³; $F(3, 35) = 4.1$, $p = 0.01$].

TH⁺ Fiber Density of the Grafts

The density of outgrowing fibers originating from the graft was evaluated and calculated in relation to the fiber density of the contralateral (intact) striatum. There was only a limited fiber outgrowth observed in all grafted groups that amounted to ~ 5 – 10% TH⁺-reinnervation with no significant differences between the groups.

Survival of 5-HT⁺ Neurons In Vivo

Immunohistochemical cell counts of 5-HT⁺ are shown in Figure 9B. The wtRH group showed 593 $\pm$

235 surviving 5-HT⁺ neurons and it was significantly higher survival than in all other groups [$F(3, 35) = 4.5$, $p = 0.009$]. In contrast, the groups tgVM, tgRH, and wtVM were not significantly different from control and sham animals.

DISCUSSION

The results of the present study demonstrate that the caudal extension of Otx2 expression and the concomitant caudal shift of the midbrain/ hindbrain boundary (MHB) in *En1^{+/Otx2lacZ}* transgenic mice increases the number and, importantly, the area of DA neurons in the mid-brain/hindbrain region (MHR). In addition, the transgenic mice showed a reduced number of 5-HT neurons. This was seen in both adult mice in situ and during fetal development, as observed in cell cultures derived from E13.5 mouse embryos. Furthermore, the selective dissection and subsequent transplantation of brain stem-derived cell suspensions revealed that the rostral hind-

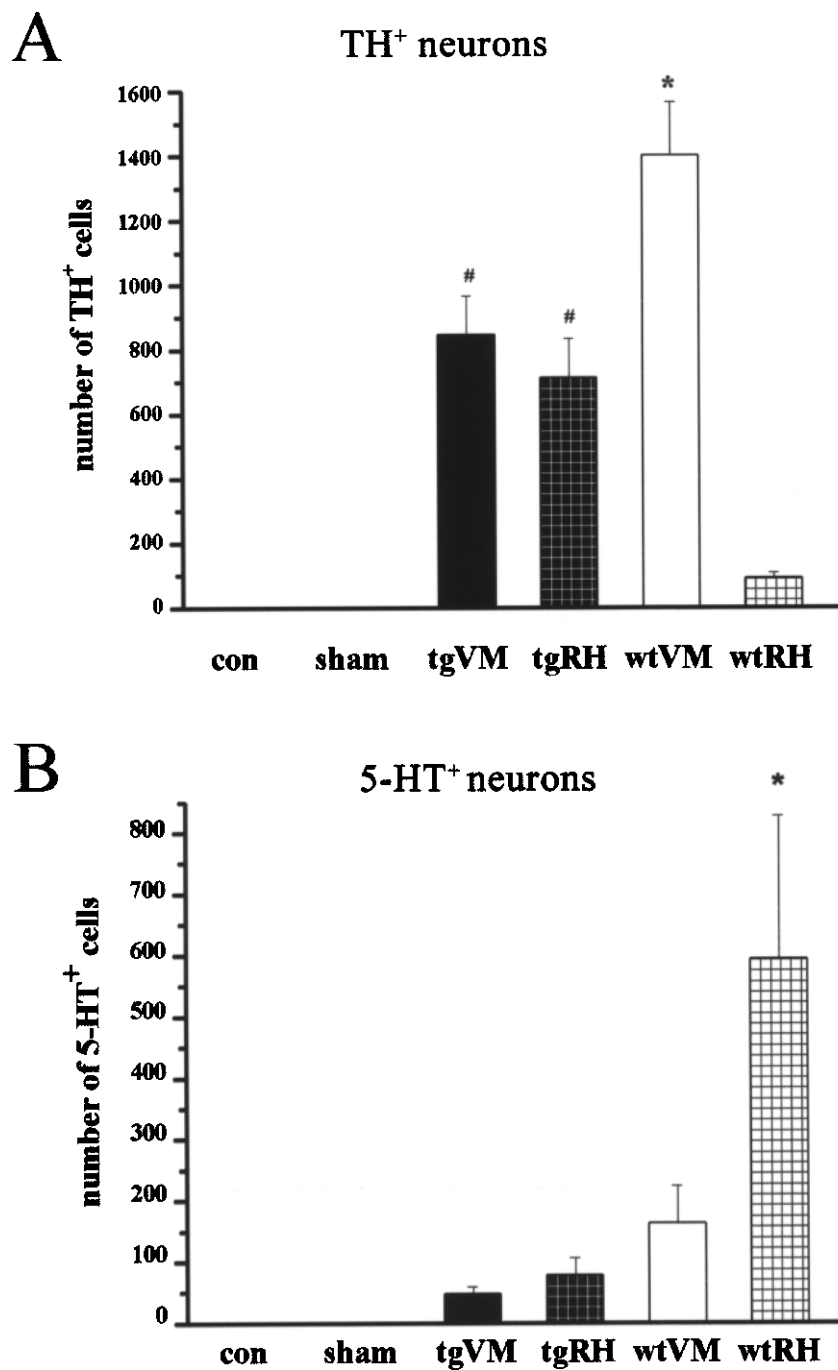


Figure 9. Number of TH⁺ (A) and 5-HT⁺ (B) neurons in the graft groups. Neither TH⁺ nor 5-HT⁺ neurons could be identified in control and sham-grafted animals. Good TH⁺ neuron survival was found in transgenic-derived grafts (tgVM: 846 ± 119 cells per graft; tgRH: 713 ± 120 cells per graft). The highest survival was found in wtVM animals with 1400 ± 165 surviving TH⁺ neurons per graft, which was significantly higher than all other groups ($p < 0.01$). A good survival of 5-HT neurons was only observed in the wtRH group with 593 ± 235, whereas 5-HT⁺ cell numbers were 163 ± 28 (wtVM), 78 ± 28 (tgRH), and 48 ± 12 (tgVM). #Significant differences from all other nontransgenic groups; *significant differences from all other groups.

brain (RH) in transgenic mice contained large numbers of DA neurons that survived and produced substantial functional recovery in 6-OHDA-lesioned rats. This was markedly different from grafts derived from the wtRH, where only a few DA neurons were found and there were no functional effects observed either in the apomorphine or in the amphetamine-induced rotation test. In line with these findings, tgRH grafts contained only a few 5-HT neurons, whereas the wtRH-grafted group contained the highest number of 5-HT neurons.

Previous studies reported that *En1*^{+Otx2lacZ} transgenic mouse embryos had an increased volume of DAT⁺ DA midbrain neurons that were distributed more caudally following the MHB shift and, thereby, extending the DA VM region (6,7). The morphological changes persisted into adulthood in the *En1*^{+Otx2lacZ} mice, which still showed a significant increase in TH⁺ volumes and cell numbers (7). Using a retrograde labeling method, the caudal, ectopically located DA neurons in the *En1*^{+Otx2lacZ} mice were also shown to innervate the striatum (7). This finding, together with an increased locomotor activity of the transgenic mice (7), suggested that the ectopic DA neurons have characteristics of substantia nigra (SN) rather than ventral tegmental area (VTA) neurons. The molecular identity of these cells as GIRK2⁺ SN neurons (36), however, has not been established so far. The underlying molecular and genetic mechanisms in the *En1*^{+Otx2lacZ} mice are extensively discussed elsewhere (33,34). However, in light of the current attempts to understand DA stem cell biology and to develop stem cell-based transplantation approaches for PD [see, e.g., (16, 23)], it became relevant to perform a comparative analysis of this ectopic DA neuron population in the RH of transgenic animals with regard to their pattern of survival and functional integration following transplantation in an animal model of PD.

The previous study by Brodski et al. (7) did not assess whether the caudal extension of the DA domain into the RH of *En1*^{+Otx2lacZ} mice could have been due to a wider distribution and thus reduced density of the TH⁺ cells in the transgenic brain. In the first part of the present study, we therefore evaluated the area of distribution, the absolute number, and the expression of TH and DAT in DA and 5-HT in serotonergic cell populations in the MHR of 2-month-old *En1*^{+Otx2lacZ} transgenic and wild-type mice. Using a well-established and unbiased stereological method and a higher number of experimental animals, we show here that the area containing DA cells was nearly twice as big as in the wild-type mice and caudally extended in the transgenic animals prolonging the distribution area of 5-HT neurons. In both studies, the increase of TH⁺ cells was about 1.2-fold of the wild-type, although in the present study this did not reach statistical significance. In addition, we could show

that 80% of the midbrain TH⁺ cells coexpressed DAT both in transgenic and wild-type mice. This consolidates the hypothesis that the caudally located TH⁺ cells in transgenic mice are phenotypically comparable DA neurons with respect to their homotopic VM-derived DA neurons counterparts.

Furthermore, the absolute number of 5-HT cells was significantly lower in the transgenic group compared to wild-type animals, which explains the observation of the significant decrease of 5-HT neurotransmitter levels in the striatum (7). In contrast, the area of distribution of 5-HT⁺ cells was not changed between the wild-type and transgenic animals. Brodski and colleagues showed that in *En1*^{+Otx2lacZ} transgenic E12.5 embryos the volume of distribution of 5-HT cells decreased significantly compared to wild-type animals. However, our results indicate that the alteration of the location of rostral 5-HT neurons observed during embryogenesis in the E12.5 *En1*^{+Otx2lacZ} transgenic old mouse embryos are not preserved in adulthood. An interesting aspect of using grafts derived from *En1*^{+Otx2lacZ} transgenic mice embryos is the potential benefit of having donor material containing a low number of 5-HT neurons. A major side effect in long-term L-DOPA therapy for Parkinson patients are dyskinesias. Furthermore, VM graft-induced dyskinesias observed in patients after transplantation of fetal VM tissue are jointly responsible for the current reluctance in clinical transplantation studies (20,45). Recent publications have shown that 5-HT neurons in VM grafts can contribute to graft-induced dyskinesias in patients (5). In addition, 5-HT neurons are known to play a major role in L-DOPA conversion to DA, as well as in DA storage and release in L-DOPA-treated Parkinson patients. Carta and colleagues could prove a causal link between 5-HT neuron-dependent DA release and abnormal involuntary movements in the rat model of PD (9). In addition, grafts rich in 5-HT neurons have been shown to increase L-DOPA-induced dyskinetic effects in the rat model of PD (8) whereas treatment with 5-HT-receptor agonists reduced L-DOPA-induced dyskinesias in primate and rat models of PD (26). While the intention of the present study was to show that TH⁺ cells derived from tgRH grafts have functional capacities similar to TH⁺ cells derived from the VM of wild-type control mice, the benefit of having access to donor material with low numbers of 5-HT neurons could, in the future, help to further elucidate the potential risks of 5-HT neurons in VM grafts.

When VM- and RH-derived cells dissected from E13.5 wild-type and transgenic embryos were taken into cell culture, a significantly higher proportion of TH⁺ cells in cultures derived from wtVM was found compared to all other groups. Cultures prepared from tgVM tissue contained higher percentages of TH⁺ cells than

cultures derived from the tgRH. Analysis of 5-HT⁺ cells revealed significantly higher percentages in wtRH-derived cells than in all other groups; this group also showed significantly lower percentages of DA cells. It has been suggested that Otx2 expression in the rostral hindbrain may cause a transformation of progenitor cell differentiation from 5-HT into DA neurons, which may, at least partly, explain the observed effects in our study (7). In order to exclude significant different apoptotic rates within the different brain stem subregions, all experimental groups were evaluated in vitro for caspase-3, but no significant differences could be found. The differences in the survival rates of DA neurons between the tgVM and tgRH were only seen in vitro and may thus relate to specific cell culture conditions other than apoptotic mechanisms, an observation that will need to be addressed in further studies.

The single cell suspensions derived from the VM of E13.5 *En1^{+/Otx2lacZ}* mice when transplanted into the rat model of PD resulted in the DA neuronal survival in the range of 2–5%. This is in accordance with previous studies and is based on the observation that only approximately 8–10% of the cells of the original cell preparation are TH⁺ (3,8,13,15,29). Similarly, the survival of 5-HT neurons derived from the wtRH is around half of that seen with VM-derived DA neurons, which has been described in the allograft transplantation paradigm by (12).

Interestingly, we observed a comparable survival rate of DA neurons that were derived from the tgVM and tgRH, but only a few DA neurons could be identified within wtRH-derived grafts. Compared with grafts derived from wtVM donor tissue, tgVM grafts seem to show a relative decrease in survival of DA neurons. However, this result corresponds to our previously discussed in vitro data, in which we could show that after 1 DIV, cultures derived from wtVM showed significantly more TH⁺ cells than all other groups. The in vitro relation of tgVM-derived to wtVM-derived DA cells after 1 DIV was identical to the relation of surviving DA cells in grafts derived from tgVM versus wtVM. The degree of graft-derived TH⁺ fiber innervation as evaluated within the whole host striatum was relatively low in our study in all groups. This might be due to the xenograft transplantation model that may result in a higher degree of immunological host responses, despite the immunosuppression, leading to decreased neuron survival and axon terminal reinnervation (25,46).

Importantly, grafts derived from the tgRH produced a similar degree of functional behavioral improvement in the drug-induced rotation test as seen in VM-derived grafts from both transgenic and wild-type animals. This is convincing evidence that these tgRH-derived DA neurons not only express the key enzyme (i.e., TH), but also

provide a functional DA release mechanism characteristic for VM-derived DA neurons. Grafts derived from wtRH did not induce this functional improvement, which can be taken as further support for this hypothesis and has been demonstrated previously (12). These novel findings need to be pursued to the level of assessments that were carried out previously for VM-derived DA neurons in order to fully elucidate their anatomical, biochemical, and functional profile [see (16)]. This may have significant implications also for the further development of stem cell-based restorative therapies for PD and, potentially, other neurodegenerative diseases (11,45).

Cell restorative therapies for PD and other neurodegenerative diseases are so far dependent on cell transplantation of fetus-derived tissue and the source of this tissue is, apart from ethical concerns, unstable and very limited (2). Embryonic and mesenchymal stem cells are highly promising alternative cell sources of DA neurons (16,18,21). Recent research has put high emphasis on understanding the exact molecular mechanisms during embryonic DA neuronal development to in vitro generate DA neurons from stem cells (19). Recently, Chung and colleagues could identify a Wnt1-Lmx1a autoregulatory loop during the DA differentiation of embryonic stem cells in vitro and in vivo. This loop directly regulates the expression of Otx2 and other signaling intermediates. By means of retroviral transduction, the authors could show that forced overexpression of Otx2, Lmx1a, and FoxA2 resulted in a robust induction of DA neuron differentiation and finally in mature DA neurons including A9-like midbrain DA neurons (10). However, it is crucial to prove that stem cell-derived DA neurons can functionally integrate and lead to a behavioral recovery in a preclinical setting before clinical trials are initiated. The rat model of PD used in the present study provides such a preclinical model. In addition, a parallel study of *En1^{+/Otx2lacZ}* and *En1^{+/-}* transgenic mice would be needed to further investigate the developmental and adult influence of Otx2 expressed under the control of the *Engrailed 1 (En1)* promoter, which may unravel a potential neuroprotective effect of Otx2.

In conclusion, we could demonstrate that the ectopic induction of DA neurons in the RH of *En1^{+/Otx2lacZ}* mice results in a novel population of DA neurons that can i) survive in a rat xenotransplantation model of PD and ii) lead to behavioral improvement in drug-induced rotational asymmetry comparable to DA neurons derived from wild-type and transgenic VM. These results highlight that cell transplantation can be used as a valuable experimental tool to investigate the restorative potential of DA neural stem cells in vivo. Modern molecular biology and histological analyses can accurately define the physiological and morphological phenotype of stem cell derived-DA neurons. However, preclinical transplanta-

tion models are crucial to further elucidate functional integration capacities of these therapeutic candidates before clinical trials are initiated. Thus, transplantation protocols, as applied in the present study, may become a standard approach for neural stem cells prior to any clinical applications in patients and, thereby, enhance both the quality standards of clinical neurotransplantation and our understanding of neural stem cell development in the engrafting environment.

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