Transplantation of the rat suprachiasmatic nucleus. Functional studies and ex vivo adenoviral vector-mediated gene transfer

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Adenoviral vector-mediated gene transfer and neurotransplantation: possibilities and limitations in grafting of the fetal rat suprachiasmatic nucleus

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Several studies have reported on the use of primary neural cells transduced by adenoviral vectors as donor cells in neurotransplantation. In the present study we examined whether adenoviral vector-mediated gene transfer could be used to introduce and express a foreign gene in solid neural transplants of fetal suprachiasmatic nucleus (SCN) tissue. A recombinant adenoviral vector containing the reporter gene LacZ encoding for β-galactosidase (Ad-LacZ) was used in order to establish the optimal procedure for ex vivo gene transfer. Expression of β-galactosidase was dependent on the duration of the infection and on the vector concentration. Infection for a short period (<4h) with a high concentration of Ad-LacZ (3.4x10^9 pfu/ml), or for 18 h with a lower vector concentration (2x10^8 pfu/ml), resulted in expression of β-galactosidase in a large number of neurons and glial cells up to 21 days in vitro. When infected fetal SCN tissue was implanted in the third ventricle of adult Wistar rats, expression was high after 8 days. After 21 days, the number of β-galactosidase expressing cells had clearly declined, but expression remained present for at least 70 days. The method described in this paper might be applicable to introduce trophic factors in SCN grafts in order to support graft survival and to stimulate neurite outgrowth.

Introduction

Animal studies have shown that transplants of fetal neural tissue have a remarkable capacity to mature, to integrate with the host brain and to promote functional recovery,
either by trophic support or by replacing lost neurons. Meanwhile, human transplantation trials gave new hope for the treatment of neurodegenerative diseases such as Parkinson's disease (PD).

The clinical symptoms of PD, caused by a loss of dopaminergic (DAergic) cells in the substantia nigra (SN) and a concomitant loss of dopamine release in the striatum, can initially be alleviated by replacement of dopamine via oral administration of the precursor L-DOPA. However, this treatment leads to serious side-effects and loses its efficacy as more and more DAergic cells in the SN die. Supplementation of DAergic neurons by means of neurotransplantation of fetal dopaminergic neurons has yielded promising results in animal models of PD, including alleviation of the induced behavioral impairments [4,5,38]. The clinical trials in human PD patients have shown that fetal mesencephalic transplants can improve motor function to some extent in a long-term fashion [19,30,35,39]. One of the major problems associated with neurotransplantation of fetal DAergic neurons, however, is the low survival rate of grafted fetal neurons [4,34].

Although the cause of the cell loss in PD and other neurodegenerative diseases is largely unknown, application of trophic factors is thought to halt or slow down the degeneration process. This is based on in vitro studies which have shown that glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), and acidic and basic fibroblast growth factor (aFGF and bFGF) can protect rat mesencephalic DAergic cells from neurotoxicity by 6-OHDA, MPP⁺ or MPTP [21,27]. The latter three trophic factors have also been shown to promote behavioral recovery after intracerebral administration in the animal models for PD (for a review see [21]). However, administration of these factors to the human brain is very difficult since these molecules do not cross the blood-brain barrier. Thus, invasive methods (repeated intracerebroventricular injections or infusion via osmotic minipumps) are necessary to administer these trophic factors to the human brain.

New developments in genetic modification of cells are proposed as a putative solution to these problems [15,37]. In particular, the use of viral vectors for gene transfer has shown great promise. The tyrosine hydroxylase (TH) gene has been introduced in mitotic cells (fibroblasts and endocrine cells) via these methods, in order to obtain DA-producing cells which are easier to obtain and more readily survive transplantation. Implantation of such cells reduces rotational behavior in parkinsonian rats [18,25]. Direct intracerebral injection of TH gene-containing vectors was applied successfully as well [16,26,28]. Not only is viral vector-mediated genetic modification a useful way to obtain specific donor cells that could take over the function of lost or dysfunctioning neurons, it can also be used to introduce neurotrophic factors in the CNS. For instance, degeneration of DAergic cells in MPP⁺- or 6-OHDA-treated rats is prevented by intrastriatal transplantation of fibroblasts infected with a retroviral vector containing the BDNF gene [20,33]. Moreover, fibroblasts genetically modified to produce bFGF and cografted with fetal DAergic neurons have been shown to increase the number of surviving DAergic neurons and to improve functional recovery in 6-OHDA-treated rats [46].
Genetically modified mitotic cells such as fibroblasts have the advantage of being produced in large quantities prior to transplantation. However, they also bear the risk of uncontrolled mitosis following transplantation. Post-mitotic fetal neuronal cells, on the other hand, are advantageous in that they can integrate into neural tissues while exhibiting fully mature neuronal properties; e.g., including neuritic outgrowth, synapse formation and calcium-mediated transmitter release. The recently developed recombinant adenoviral and adeno-associated viral vectors are able to transduce post-mitotic nerve cells [2,3,14,28,31]. To date transplantation of primary cultured neuronal cells genetically modified with adenoviral vectors carrying the bacterial reporter gene LacZ have been reported and have shown that expression can be retained for weeks [43] or months [41]. No studies have, as yet, been reported on viral vector-mediated gene transfer in solid piece fetal grafts.

In the present study, rat fetal suprachiasmatic nucleus (SCN) tissue pieces were used to explore procedures for gene transfer by adenoviral infection prior to grafting. Transplantation of this circadian rhythm-generating nucleus into the third ventricle of arhythmic, SCN-lesioned adult rats or hamsters re-instates circadian drinking and locomotor rhythms [8,22,32,40]. However, the percentage of rats recovering circadian rhythmicity is relatively low (about 40%). This is probably due to limited reinnervation of the host brain by the graft [1,9,22,45,47]. Adenoviral vector-directed expression of the appropriate neurotrophic factor in the SCN graft could potentially improve functional restoration due to enhanced neurite outgrowth. As a necessary first step to establish gene transfer in fetal SCN grafts, gene transfer was performed using a prototype adenoviral vector (Ad-LacZ) containing the LacZ reporter gene encoding β-galactosidase (β-gal). Effects of infection time and adenoviral vector concentration on the expression of β-gal were investigated. In addition, time courses of transgene expression during in vitro culturing, and following transplantation, were compared.

Materials and methods

Construction of recombinant Ad-LacZ

The recombinant adenoviral vector containing the Escherichia coli LacZ gene encoding for β-gal was generated as described by Hermens et al. [24]. In short, a construct containing a CMV promoter/enhancer, the LacZ gene and a SV40 poly(A) sequence was inserted into an adenoviral targeting plasmid pAd309dEl.s1. The plasmid was linearized with Sall and cotransfected with Clal and Xbal-truncated Ad5d/309 viral DNA in 911 cells [17]. Recombinant plaques were propagated in 911 producer cells and purified by double CsCl banding. Purified recombinant adenovirus (Ad-LacZ) was dialyzed against TS (25 mM Tris, 137 mM NaCl, 6 mM KCl, 0.7 mM Na2HPO4, 1.1 mM MgCl2, pH 7.5) and stored at -80°C in TS containing 10% glycerol until use. Titers of the adenoviral vector stocks were determined by plaque assay on 911 cells. Titers are expressed as plaque forming units per ml (pfu/ml). Three different adenoviral stocks were used, with titers ranging from 2x10^8 pfu/ml to 1.7x10^11 pfu/ml.
Tissue preparation and viral infection
Fetal SCN tissue was obtained from 17-day-old Wistar rat embryos. After decapitation of the dam the uterine horns were removed and kept on ice. Fetal brains were dissected one by one under a stereomicroscope. An approximately 1 mm thick coronal slice was made of the anterior hypothalamus by free-hand sectioning [7] and transferred into a large drop of Hank’s balanced salt solution supplemented with 0.15% glucose. A triangular part of the basal hypothalamus was dissected, including the SCN, adjacent hypothalamic tissue, optic chiasm, and a part of the ependymal lining of the third ventricle. The total volume of the tissue was approximately 1 μl. The tissue was cut into 8-10 smaller pieces and transferred to a 96-well flat-bottom culturing plate (Nunc, Roskilde, Denmark) containing 50 μl of chemically defined medium per well (R16; [42]. The medium contained either the Ad-LacZ vector or an equivalent amount of the vehicle. The plates were placed in a metal grid box at 100% humidity, 37°C and 5% CO₂ for 18 h unless stated otherwise, after which the medium was removed and the tissue rinsed twice with R16. The SCN tissue of two fetuses was pooled and transferred to a 25 mm petri dish (Nunc, Roskilde, Denmark) containing 1.5 ml R16. These free-floating preparations of infected SCN tissue were either cultured in vitro for 8-10 days (unless stated otherwise) under the same conditions (half of the medium changed twice a week), or transplanted to the bottom of the third ventricle of male Wistar rats within 5 h after the vector incubation.

Grafting
The grafting procedure has been described previously [22]. Wistar rats weighing 230 to 300 g were anesthetized with Hypnorm (0.1 ml i.m. per 100 g of body weight; 10 mg fuanisone and 0.315 mg fentanyl citrate per ml; Janssen Pharmaceutica, Tilburg, The Netherlands) and placed in a David Kopf stereotactic apparatus. The skull was exposed and a hole was drilled at 1.4 mm anterior and 1.0 mm lateral to the bregma. Infected or non-infected SCN tissue derived from two fetuses (total volume of approximately 2 ul) was taken up in a metal implantation canula (OD 0.9 mm, ID 0.6 mm) which was mounted on the stereotactic frame at a lateral angle of 10°. The canula was lowered through the hole and the tissue was slowly ejected at 8.5 mm below the dura. The canula was left in place for 1-2 min after each ejection and then slowly withdrawn from the brain.

Experimental design
EFFECT OF INFECTION TIME ON TRANSGENE EXPRESSION (EXPERIMENT 1)
SCN tissue was incubated with Ad-LacZ for 0.5, 1, 4, 8 or 18 h in 50 ml R16. The experiment was performed with final concentrations of 2x10⁶ and 3.4x10⁹ pfu/ml (experiment 1A and 1B respectively). The culturing period was 8-10 days in vitro (DIV). Expression of β-gal was visualized using X-Gal histochemistry in experiment 1A and by means of immunocytochemistry in experiment 1B (see below).
TIME COURSE OF TRANSGENE EXPRESSION IN VITRO (EXPERIMENT 2)
SCN explants were cultured for 8 and 21 days (experiment 2A) or for 2, 4, 8 and 21 days (experiment 2B) after 18 h incubation with $2 \times 10^8$ and $3.4 \times 10^9$ pfu/ml respectively. Expression of β-gal was visualized with X-Gal histochemistry or immunocytochemistry as in experiment 1.

EFFECT OF VIRAL CONCENTRATION ON TRANSGENE EXPRESSION (EXPERIMENT 3)
SCN tissue was infected for 18 h with different virus concentrations. In experiment 3A, explants were infected with $2 \times 10^8$ and $1 \times 10^9$ pfu/ml, and expression of β-gal visualized at 8 DIV using X-Gal histochemistry. In experiment 3B, tissue was infected with vector concentrations ranging from $7 \times 10^8$ to $1.7 \times 10^9$ pfu/ml and β-gal was visualized at 10 DIV by means of immunocytochemistry.

TIME COURSE OF TRANSGENE EXPRESSION IN TRANSPLANTS (EXPERIMENT 4)
SCN tissue was incubated for 18 h in $4 \times 10^6$ or $2 \times 10^8$ pfu/ml Ad-LacZ in experiment 4A and 4B respectively. The explants were subsequently grafted into the third ventricle of adult Wistar rats (see below). In experiment 4A the animals were sacrificed 8 days post-grafting (DPG) and expression of β-gal was visualized using X-Gal histochemistry. In experiment 4B, SCN tissue was used for both grafting and in vitro culturing to compare directly the time course of β-gal expression in vivo and in vitro. Rats were sacrificed at 8, 21 or 70 DPG and cultures were fixed at 8 and 21 DIV. Control grafts were incubated with R16 with vehicle prior to grafting. Both cultured and grafted SCN tissue was immunocytochemically stained for β-gal and for vasoactive intestinal polypeptide (VIP), a marker peptide for SCN neurons.

Tissue treatment and staining procedures

IN VITRO CULTURES
At the end of the culturing period, SCN cultures were fixed for 2 h with 4% paraformaldehyde (PF) in 0.1 M sodium phosphate buffer pH 7.4 (PB) and rinsed with 0.1 M sodium phosphate-buffered saline pH 7.4 (PBS). Cultures of experiments 1A, 2A and 3A were subsequently stained for β-gal activity using the chromogen X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) as a substrate. Whole explants were immersed for 3 h at 37°C in 1 mg/ml X-Gal (dissolved as 1 mg/25 μl DMSO), 5 mM K$_3$Fe(CN)$_6$, 5 mM K$_4$Fe(CN)$_6$, and 2 mM MgCl$_2$ in PBS. The tissue of experiment 1A was then rinsed for 2 h with PBS and kept overnight at 4°C in PBS containing 15% sucrose. Sections (20 μm) were cut using a cryostat. The X-Gal-stained cultures from experiments 2A and 3A were embedded (together with a piece of fixed adult cerebral cortex in order to keep track of the sections after vibratome sectioning) in 10% gelatin in 0.1 M Tris/HCl-buffered saline pH 7.6 (TBS) of 50°C. The gelatin was solidified at 4°C, after which 4% PF in PB was added for 24 h postfixation at 4°C in order to achieve adherence of tissue and gel. Gelatin blocks including the cultures were serially cut in 50 μm vibratome sections, which were evaluated for X-Gal staining.
The cultures from experiments 1B, 2B, 3B and 4B were embedded in gelatin immediately following 2 h of fixation and rinsing with TBS. Two alternate sets of 50 µm vibratome sections were collected, one of which was stained immunocytochemically for β-galactosidase in TBS containing 0.25% gelatin and 0.5% Triton X-100. The free-floating sections were first rinsed and incubated overnight at 4°C with a monoclonal mouse β-gal antibody (GAL-13; 1:2000, Sigma Immuno Chemicals, St. Louis, MO) followed by 1 h incubation with the secondary antibody coupled to horseradish peroxidase (rabbit-anti-mouse IgG; 1:100, DAKO, Glostrup, Denmark). The final step was incubation with 0.5 mg/ml diaminobenzidine (DAB) and 0.2% ammonium nickel sulphate in PBS. Within one experiment the incubation time with DAB was kept constant to allow for quantification.

**IN VIVO TRANSPLANTS**

Grafted animals were sacrificed through intracardiac perfusion with ice-cold saline followed by 4% PF in PB. The brains were removed and a 1 cm thick coronal slice was made with its frontal limit at the optic chiasm and its caudal limit at the posterior hypothalamus, thus including the third ventricle and the graft. In experiment 4A this slice was postfixed for 2 h, rinsed for 1 h in PBS, immersed in the X-Gal solution as described above for 4 h at 37°C and rinsed again. Brain slices were then vacuum-embedded in a 10% gelatin solution in TBS as described by Griffioen et al. [23]) to keep the ventricular graft in place during sectioning. The gelatin block was postfixed for 24 to 36 h with 4% PF in PB and cut into 50 µm coronal vibratome sections. For experiment 4B, the coronal brain slice was postfixed for 48 h and thoroughly rinsed for 24 h in TBS, embedded in gelatin and sectioned as in experiment 4A. Alternate sections, pretreated with 3% hydrogen peroxide in TBS for 10 min, were immunocytochemically stained for β-gal (see above) and VIP.

VIP was detected with a polyclonal rabbit antibody (VIPER; 1:2000 [10]), followed by 1 h incubation with goat-anti-rabbit IgG (Betsy; 1:100) and a 1 h incubation with a rabbit peroxidase-antiperoxidase complex (PAP; 1:1000). The antibody complexes were visualized with DAB in the presence of 0.2% ammonium nickel sulphate.

**Quantification**

X-Gal histochemistry resulted in a blue staining pattern while immunocytochemistry with DAB with nickel enhancement resulted in a purple staining. A semiquantitative evaluation of the amount of β-galactosidase staining was accomplished by scoring the staining intensity on every other section by two independent observers. Staining was scored between + (only a few cells expressing β-gal) and +++++ (explant nearly completely filled with the staining product). For all groups, the median value of these scores is given.

In addition, for the explants the total area of the stained structures was measured in every other section using a computerized image analysis system equipped with a high resolution monitor and connected to a slow scan high resolution colour camera (KAT-IBAS/Progres 3010 system, Kontron) mounted on an Axioskop microscope (Zeiss). Using a 5x or 10x objective each section was loaded as a 2480 x 2240 pixel image. In this image the explant...
was outlined manually and a binary mask covering all positively stained structures was calculated on the basis of the ‘red’ image of the original color (RGB)-image. The algorithm used to calculate this mask consisted of two steps: extraction of the small structures (lowpass filtering with a 10 x 10 matrix followed by a minimum filter and image segmentation), and extraction of large stained areas by dynamic segmentation. The total area was measured in the binary image resulting from the merging of the small structure- and the large-structure binary images. From the total and mask areas in a series of sections from the same explant, estimates of the total volume of the explant and the total volume of the stained structures were calculated. The results are expressed as average percentage stained volume area.

Results

Transgene expression in vitro

In vitro infection of small pieces of fetal rat SCN tissue with Ad-LacZ resulted in pronounced cellular expression of β-gal in all cultures. From 8 DIV onwards the cultures consisted of clustered pieces of tissue in which the β-gal-stained cells were not evenly distributed, but often found in clusters (Fig. 1A). Staining was present in cell bodies and cellular processes, and often the typical morphological appearance of neurons and glial

![Figure 1. Infection of rat fetal SCN explants with Ad-LacZ.](image)

(A) Low power photograph of an explant infected for 18 h and immunocytochemically stained for β-gal at 8 DIV. The small pieces have clumped together to form bigger pieces. The β-gal-positive cells are distributed in groups. (B) Higher magnification of the explant shown in (A). Immunoreactivity is seen in cell bodies and cellular processes of neurons (n), glial cells (g) and endothelial cells (e). (C) High magnification of an explant infected under the same conditions as (A/B) but stained histochemically with X-Gal. The stained structures have a ruffled appearance and the fine cellular processes are less well visible as compared to B. Scale bar = 400 µm (A), 40 µm (B and C).
Table 1. Summary of the conditions used in the in vitro experiments and the resulting β-galactosidase expression in the SCN explants.

<table>
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<th>days in vitro</th>
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N, number of explants. Scores are mean values and indicate: +, a few scattered cells expressing β-gal; ++++++, explant nearly completely filled with the staining product. ++, +++ and ++++ represent a degree of expression in between these two ratings.

cells could be distinguished (Fig. 1B). When a portion of the ventricular wall was present in the explant, the ependymal cells were preferentially infected (Fig. 1B). Immunocytochemical detection of β-gal resulted in a much better resolution of the fine cellular processes of the transduced cells (Fig. 1A/B) as compared to histochemical detection (Fig. 1C). This is probably due to diffusion of the reaction product formed during the enzyme histochemical staining procedure.

Effect of time of infection on transgene expression
Using an adenoviral vector concentration of 2x10⁸ pfu/ml (experiment 1A), X-Gal histochemistry revealed a low number of β-gal-expressing cells at 8 DIV for infection periods up to 4 h, whereas 18 h of infection resulted in significantly more β-gal positive cells in the explants (Table 1; Fig. 2A,B,C). Percentages of the volume of the stained structures varied between 0.8 and 2.4% for explants infected for 1 to 4 h, and increased to
11% in explants infected for 18 h (Fig. 3). The effect of infection time was less pronounced when the tissue was infected with a higher concentration of the viral vector (3.4x10^6 pfu/ml; experiment 1B). An infection of 1 h resulted in numerous cells expressing β-gal at 10 DIV, whereas infection for 4, 8 and 18 h resulted in a larger number of β-gal-positive cells (Table 1; Fig. 2D,E,F).

**Time course of transgene expression in vitro**

Microscopical observations (Table 1) and quantitative data of stained volume area of the explants (Fig. 4) showed that, after incubation with 2x10^8 pfu/ml (experiment 2A), the number of β-gal-expressing cells at 8 and 21 DIV was similar. At 21 DIV, however, the X-Gal staining revealed dark spots on the outer rim of the explants in which cell morphology was difficult to distinguish, while in the core of the explants staining was absent (Fig. 5D). This probably was due to central necrosis, which was also observed in control explants (not
shown) and is, therefore, probably related to the present culture conditions.

Infection with a higher vector concentration (3.4x10⁹ pfu/ml; experiment 2B) resulted in a similar number of immunocytochemically stained cells at 2, 4, 8 or 21 DIV (Table 1). Computerized analysis of the stained volume areas did not reveal differences in the total amount of staining (Fig. 4). A difference could be seen, however, in the morphology of the stained structures. In the 2 DIV explants, β-gal positive cells exhibited good morphology (Fig. 5A), whereas at 8 and 21 DIV the extent of the cell processes was reduced (Fig. 5B). At these time points areas with central necrosis also became visible in which the morphology of the stained structures was hardly recognisable (Fig. 5C).

**Effect of the concentration of the viral vector on transgene expression**

In experiment 3A the explants were incubated with 2x10⁸ or 1x10⁹ pfu/ml Ad-LacZ, which in both cases resulted in a profound X-Gal staining after 8 DIV. Although the percentages of the blue stained volume area were similar (Fig. 6), a difference could be seen in the morphology of the stained structures. The lowest concentrations gave a dense staining, which was often distributed in clusters with cells showing a healthy, well defined morphology. Incubation with the highest concentrations resulted in similar clusters, which were mainly located at the border of the explant and had a higher staining intensity. Some cells still showed stained processes, but many cells had clearly deteriorated, and dot-like structures were visible. In experiment 3B a clear difference was visible in the amount of β-gal expression in explants infected with 7x10⁸ or 1.7x10¹⁰ pfu/ml Ad-LacZ (Fig. 6). Immunocytochemistry gave an intense staining in the center of the explants that were treated with the highest concentration of viral vector. The sites of high β-gal expression often coincided with sites of necrosis.
Figure 5. Time course of β-gal expression in Ad-LacZ-infected fetal SCN explants. (A) Infection with $3.4 \times 10^9$ pfu/ml for 18 h gave a high expression at 2 DIV and a good morphology of the immunocytochemically stained cells. (B and C) As the culturing period increases to 8 and 21 DIV the staining remains visible, but central necrosis becomes visible (arrows). (D) Explants stained at DIV 21 using X-Gal histochemistry showed staining mainly at the borders, but no β-gal activity in the central necrotic site (infection with $2 \times 10^8$ pfu/ml). Note that at DIV 21 (C,D) the morphology of the cells is changed and that cellular processes are hardly recognisable. Scale bar = 100 μm.
Time course of transgene expression in transplants

Intracerebroventricular transplants of fetal SCN tissue infected for 18 h with 4x10^6 pfu/ml exhibited few X-Gal-stained cells (Fig. 7A). In experiment 4B the procedure was repeated with a concentration of the viral vector (2x10^6 pfu/ml) which had previously shown long term expression without toxic effects in the in vitro experiments. Transgene expression in the implants was evaluated with β-gal immunocytochemistry and was directly compared with that of parallel explant cultures. At 8 and 21 DIV a similar high level of expression was found as observed in experiment 2A (Table 1), whereas the transplanted tissue exhibited abundant expression of β-gal at 8 DPG, and a reduced number of β-gal positive cells at 21 or 70 DPG (Fig. 7B-D; Table 2). The staining of β-gal was present in both neuron- and glial-like cells which had a healthy appearance (Fig. 7D).

VIP was barely visible in 8 DPG implants and could only be observed in 2 out of 3 control transplants and in 3 out of 6 infected transplants. In all 21 and 70 DPG transplants, one or more VIPergic cell clusters could be recognised with an extensive network of fibers throughout the transplant (Fig. 7E). The cytoarchitecture of the cell clusters was comparable to that of the VIP staining of the host SCN. VIP staining had increased in the 70 DPG transplants as compared to the 21 DPG transplants (Table 2).

At 8 DPG, most of the infected and control transplants showed central necrosis which had disappeared at 21 DPG. 21 and 70 DPG implants had increased in volume, revealed no necrotic sites and occupied large parts of the third ventricle.

Discussion

Recombinant adenoviruses have been shown to be efficient gene transfer vectors for neural cells in vitro and in vivo [2,3,12,14,31]. Recently, adenoviral vector-directed expression of a foreign gene has been documented in dissociated transplants infected ex vivo [31,43]. The present study shows, for the first time, that solid fetal CNS explants, genetically modified with an adenoviral vector encoding the reporter gene LacZ, maintain transgene expression upon transplantation. Ex vivo adenoviral vector-mediated gene transfer in small pieces of SCN resulted in transgene expression in numerous neuronal and non-neuronal cells throughout the explant up to 21 DIV. In similarly treated transplants placed
Figure 7. Expression of β-gal in Ad-LacZ-infected fetal SCN tissue either grafted or cultured. (A) Infection with a low concentration Ad-LacZ (4x10^6 pfu/ml) prior to transplantation resulted in a few X-Gal-stained cells at 8 DPG. (B) β-Gal immunocytochemistry of a 8 DPG implant infected with 2x10^8 pfu/ml revealed a high expression of β-gal but central necrosis is seen (asterisk). (C and D) At 21 and 70 DPG the expression of β-gal is clearly diminished but the remaining cells have a healthy appearance. (E) VIP immunostaining of the DPG 70 transplant of (D) reveals clusters with VIP neurons in a more caudal section of the implant (arrows) and VIPergic fibers that have grown throughout the transplant. (F) For comparison with (C), a DIV 21 explant infected under identical conditions is shown with a profound β-gal staining and a good morphology of the cells. T, transplant; 3V, third ventricle. Scale bar = 200 μm (A-E), 100 μm (F).
Table 2. Summary of the results of experiment 4 in which Ad-LacZ-infected SCN tissue is transplanted in the third ventricle of adult rats.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Ad-LacZ (pfu/ml) in vitro explant</th>
<th>in vivo implant</th>
<th>vb-Gal</th>
<th>n</th>
<th>DPG</th>
<th>vb-Gal</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A</td>
<td>4x10⁶</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>4B</td>
<td>2x10⁸</td>
<td>4</td>
<td>8</td>
<td>+++</td>
<td>6</td>
<td>8</td>
<td>+++</td>
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<tr>
<td></td>
<td>11</td>
<td>21</td>
<td>+++</td>
<td>6</td>
<td>21</td>
<td>7</td>
<td>+</td>
</tr>
</tbody>
</table>

Expression of β-gal upon transplantation is compared to expression in in vitro explants. n, number of explants/implants; DIV, days in vitro; DPG, days post-grafting; +, a few scattered positively stained cells; ++++, large groups of cells stained for β-gal or VIP.

in the third ventricle of adult recipient rats, transgene expression persisted up to 70 days post-grafting. However, the number of transduced cells which continued to express the transgene in these transplants decreased significantly between 8 and 21 DPG.

Efficient adenoviral vector-mediated gene transfer and persistent β-gal expression in SCN explants in vitro

The efficiency of Ad-LacZ mediated-gene transfer in SCN explants depends on the concentration of the viral vector used for infection and on the time of infection. Observations in 8 DIV explants indicate that the combination of a high concentration of viral vector (3.4x10⁹ pfu/ml) with a short infection time (2 h) or a lower vector concentration (2x10⁸ pfu/ml) with a longer infection time (18 h) both result in relatively efficient gene transfer in SCN explants. Optimal infection of monolayers of cultured cells can normally be achieved with adenoviral vectors within 2 hours of exposure to the viral vector (Hermens and Verhaagen, unpublished observations). Our data indicate that solid pieces of SCN tissue can be infected within this incubation time, but that a high concentration of the vector is required to achieve infection of a large number of cells. Longer incubation times (18 h) probably allow for better penetration of the vector in the tissue pieces and thus in a larger number of β-gal positive cells in the explant, even with relatively low viral vector concentrations.

Long incubation periods with viral vector concentrations higher than 10⁹ pfu/ml resulted in the occurrence of β-gal positive cells with less cellular processes and in the occurrence of β-gal positive dot-like structures at 8 DIV. This may indicate that under these conditions the vector may have some toxic effects, an observation reported before in dissociated and explant cultures of adenoviral vector-transduced neural cells [12,43,48]. The presently used multiplicity of infection (MOI), even with the highest concentration viral vector, was not extremely high in our explants. The number of cells present in the tissue, estimated on the basis of its volume (1 μl) and DNA content (3 μg DNA/mg tissue [6]), is approximately
Optimization of gene transfer

5x10^3. When this tissue is incubated with 50 µl of the viral vector with a concentration of 3.4x10^9 pfu/ml, the MOI will be about 350. It is conceivable, however, that the population of cells located on the outside of the tissue pieces are exposed to a much higher MOI, while cells located in the interior of the explants will encounter a lower MOI and express the transgene for longer periods. The use of concentrations up to 2x10^8 pfu/ml results in a high level of expression of the transgene in numerous cells without signs of toxicity at 8 DIV. These conditions of viral vector application were employed for the transplantation experiment.

In 21 DIV explants, β-gal positive cells with extensive processes, as well as β-gal positive ependymal cells, were observed in all conditions examined, indicating long term expression. However, in all explants, control, as well as infected, loss of cellular processes and central necrosis was also visible. This necrosis was probably related to the culturing conditions rather than the presence of the viral vector, since these features were also seen in the control explants.

Long-term, but reduced, adenoviral vector-mediated transgene expression following transplantation

Upon transplantation of ex vivo Ad-LacZ-infected SCN tissue, expression of β-gal in 8 DPG transplants was comparable with the β-gal expression in the 8 DIV explants. At 21 and 70 DPG the transgene expression was drastically reduced, but a small number of β-gal-expressing cells persisted in the transplants. This loss of β-gal expression in vivo might be due to: (a) general cell loss, (b) specific cell loss due to an immunological response, or (c) downregulation of the CMV promoter which is used to drive the expression of β-gal.

A general cell loss is often observed in neurotransplantation. It was seen as central holes in both control and infected 8 DPG transplants and was, therefore, not related to the presence of the viral vector. The sites of necrosis were surrounded by cellular debris in which macrophages were present (Boer and Van Esseveldt, manuscript in preparation). This cell loss can explain the loss of β-gal-expressing cells only partially because considerable portions of the SCN grafts survived transplantation. Moreover, in the 21 and 70 DPG transplants VIP-positive SCN neurons were present in cell clusters which were comparable to the VIPergic cell clusters that were described previously [9,22,47]. Thus, the SCN neurons did not appear to be affected by the 18 h in vitro incubation prior to grafting, nor by the Ad-LacZ treatment.

The second possibility for the loss of β-gal positive cells, which can also explain the difference between the number of β-gal-expressing cells in the explants compared to the implants, is a selective cell death of the Ad-LacZ-infected cells as a result of an immune response. Direct injections of adenoviral vectors into the brain results in an intraparenchymal invasion of macrophages and cytotoxic T-lymphocytes in the area of injection [11] (Hermens and Verhaagen, unpublished results). It was also shown that viral vector injections in immuno-suppressed rodents resulted in elongated expression periods of the transferred gene [13,29]. Although the local concentration of viral particles in the host brain is low in the present study, as compared with direct injections into the brain, an immune
response directed against viral proteins present on the transduced cells is not inconceivable. This is currently under investigation.

The third possibility, a down regulation of the viral CMV promoter was suggested before [44]. This down regulation might, in addition to an immune response, be responsible for the loss of cells expressing the transgene, which is reported in studies using viral promoters [11,12,14,31,41,43]. Preliminary observations of Lowenstein et al. [36] suggest that the Rous Sarcoma Virus (RSV) promoter would be a more efficient transcriptional activator in neurons. Thus, the use of this promoter, rather than the CMV promoter, might result in prolonged transgene expression in neurons.

**Concluding remarks**

Despite the relatively low number of cells expressing the foreign gene in the SCN graft at 21 and 70 DPG, adenoviral vector-mediated gene transfer might be a valuable method to improve the function of fetal transplants. Ex vivo gene transfer in a fetal transplant with a viral vector encoding a gene for a survival or outgrowth-promoting factor (e.g., a neurotrophin gene) would result in local production of the growth factor in the transplants, thus supporting the survival and outgrowth of neurons. The SCN neurons in the fetal basal hypothalamic grafts could be stimulated in this way to enhance their neuritic outgrowth towards denervated SCN target sites in SCN-lesioned rats. This might improve restoration of the lost circadian rhythmicity. In analogy, this approach may also be useful in the development of better grafting techniques in animal models for Parkinson’s disease. High numbers of DAergic neurons die after transplantation, which can be prevented by trophic factors like BDNF and GDNF (see Introduction). Introduction of genes for these factors by means of adenoviral vectors could perhaps prevent cell death and promote functional integration of the transplant with the host brain.

**References**


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Long-term transgene expression in fetal rat suprachiasmatic nuclei following ex-vivo adenoviral vector-mediated gene transfer

Chapter 5