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Research report

# Efficient adenoviral vector-directed expression of a foreign gene to neurons and sustentacular cells in the mouse olfactory neuroepithelium

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**Abstract**

Replication deficient recombinant adenoviral vectors are efficient gene transfer agents for postmitotic cells, including neurons and glial cells. In this paper we have examined the effectiveness of adenoviral vector-mediated gene transfer to the olfactory epithelium of adult mice. We show that Ad-LacZ, a prototype first generation adenoviral vector containing an expression cassette for the reporter gene *LacZ*, directs transgene expression to mature and immature olfactory neurons and to sustentacular cells. The technique to apply the vector to the nasal cavity and the amount of viral vector per mouse are important variables that determine the success of viral vector-mediated gene transfer to the mouse olfactory neuroepithelium. Slow infusion of the viral vector solution in fully anaesthetized mice yields the best result in terms of the number of epithelial cells transduced. Infection of the olfactory neuroepithelium with a moderate amount of viral vector ($10^9$ plaque-forming units (PFU)) results in transgene expression in many cells throughout the epithelium for 8–12 days, followed by a decline in transduced cells at 25 days postinstillation of the virus. This decrement in transgene expression is consistent with the natural turnover process that occurs in the epithelium throughout adulthood. At high viral loads ($1.3 \times 10^{10}$ PFU) extinction of transgene expression occurs as early as 8 days postinjection and is accompanied by epithelial degeneration indicating that the vector dose used should be carefully chosen. Taken together, the current observations demonstrate that adenoviral vectors are effective tools to genetically modify the adult mouse olfactory neuroepithelium in vivo.

*Keywords:* Adenoviral vector; Gene transfer, in-vivo; Olfactory system; Olfactory marker protein; B-50/GAP-43

## 1. Introduction

Neurons of mammalian nervous systems are usually not replaced if they are lost in adulthood. The olfactory neuroepithelium of adult mammals is an unique neural tissue since it exhibits continuous replacement of dying neurons formed from a compartment of dividing stem cells located in the basal region of this epithelium [23,39]. The molecular anatomy of the olfactory neuroepithelium has been investigated in great detail. A number of olfactory-specific gene products that are preferentially expressed in olfactory neurons have been identified, including olfactory marker protein (OMP) [33,45], $G_{olf}$, a G-protein expressed in olfactory neurons [26,36] and a myriad of olfactory receptors [5,42,55]. Immunohistochemical studies have revealed the continued expression of neural growth-associated proteins (e.g., MAP-5 [58] and B-50/GAP-43 [57]) and a cell-adhesion molecule characteristic of immature neurons (E-NCAM) [38] in the basal half of the olfactory neuroepithelium, a region that contains immature olfactory neurons and neuronal stem cells.

The precise molecular regulation of olfactory neuron turnover, signal transduction and odor detection by primary olfactory neurons have so far mainly been examined in vitro in embryonic or postnatal cell cultures of olfactory tissues [8,41,46,47] and in membrane preparations of olfactory cilia [2,4,44]. The recent demonstration of efficient direct in-vivo gene delivery to neurons using recombinant replication deficient adenoviral vectors [1,3,13,31] encourages the idea that direct gene transfer to the mature olfactory epithelium in intact animals might be a powerful

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approach to investigate the role of specific proteins in neurogenesis, maturation, odor detection and olfactory neuron death in an in-vivo context. The purpose of the present study was to explore the ability of replication deficient adenoviral vectors to direct the expression of a foreign gene to cells of the olfactory epithelium.

We demonstrate that infusion of a recombinant adenoviral vector encoding the *Escherichia coli* β-galactosidase (*LacZ*) gene directs efficient β-galactosidase (β-gal) expression to olfactory neurons and to sustentacular cells. Transgene expression occurred predominantly in mature, OMP-expressing neurons and was occasionally observed in immature B-50/GAP-43 positive neurons. No transgene expression was apparent in stem cells in the basal region of the epithelium. These observations suggest that the viral vector is preferentially entering cells that are in direct contact with the mucus membrane covering the olfactory neuroepithelium. The establishment of efficient transfer of a foreign gene to the olfactory neuroepithelium is a first step towards in-vivo genetic intervention studies of this neuroepithelium in adult mice and may be instrumental in the elucidation of molecular mechanisms that govern olfactory neuron turnover and function.

## 2. Materials and methods

### 2.1. Viral vector construction

The recombinant replication deficient adenoviral vector containing an expression cassette for the *LacZ* gene was generated as follows. First a plasmid was constructed containing the adenovirus type 5 (Ad-5) inverse terminal repeat (ITR, adenovirus map units 0–1.25) and a region of the adenovirus genome ranging from 9.2 to 15.5 map units (pAd309dE1.sl). A human cytomegalovirus immediate early (CMV) promoter-*LacZ* reporter gene construct, linked to a SV40 poly(A) sequence was cloned in between map units 1.25 and 9.2 resulting in plasmid pAdLacZ. In order to generate recombinant Ad-LacZ virus, pAdLacZ was linearized with *Sal*I, an unique restriction site at the 5′ side of the ITR, and transfected into 911 cells [17] together with *Cla*I and *Xba*I truncated Ad5*dl*309 genomic DNA. Recombinant adenoviral plaques were isolated, propagated in 911 cells and purified by double CsCl banding as described [22]. Purified recombinant adenovirus was extensively dialyzed against TS (25 mM Tris, 137 mM NaCl, 6 mM KCl, 0.7 mM $Na_2HPO_4$, 1.1 mM $MgCl_2$, 0.9 mM $CaCl_2$; pH 7.5) and subsequently stored until use in TS containing 10% glycerol (TS/GLYC) in 30 μl aliquots at −80°C. Virus stocks were titered by plaque assay on 911 cells [17,22]. Titers are expressed as plaque forming units (PFU) per ml.

### 2.2. Animals and surgery

Five-month-old male FVB/N mice (weighing approximately 30 g; $n = 41$), obtained from the breeding facility of the Academic Medical Center in Amsterdam, were used throughout the experiments. For rapid injections the viral vector was supplied by a single and manual injection in the nasal cavity. Animals were lightly anaesthetized with ether and a dose of $2.5 \times 10^{10}$ PFU in 100 μl TS/GLYC or vehicle alone was injected in the right nostril using a 30-ga blunt needle. The needle was inserted into the nostril to a depth of 7 mm. In this position the needle does just not touch the olfactory epithelium.

Since rapid manual infusion of virus in the nasal cavity resulted in relatively inefficient transduction of the *LacZ* gene, experiments were performed to improve the efficiency of adenovirus-mediated gene transfer by testing the effect of slow infusions of the viral solution in the nasal

Table 1
Summary of the results of adenoviral vector-directed expression of β-gal in the olfactory neuroepithelium

| Infusion | Number of mice | Dose (PFU/mouse) | Days postinfusion | β-gal exp. in OE | Epithelial thickness |
|---|---|---|---|---|---|
| Rapid | 4 | $2.5 \times 10^{10}$ | 3 | + | N |
| Rapid | 3 | TS/GLYC | 3 | − | N |
| Slow | 4 | $1.3 \times 10^{10}$ | 3 | + + + | N |
| Slow | 4 | $1.3 \times 10^{10}$ | 8 | + | T |
| Slow | 3 | $10^9$ | 1 | + + + | N |
| Slow | 3 | $10^9$ | 3 | + + + | N |
| Slow | 4 | $10^9$ | 8 | + + + | N |
| Slow | 3 | $10^9$ | 12 | + + + | N |
| Slow | 2 | $10^9$ | 25 | + | N |
| Slow | 3 | $2.5 \times 10^8$ | 1 | + + | N |
| Slow | 1 | $2.5 \times 10^8$ | 3 | + + | N |
| Slow | 4 | $2.5 \times 10^8$ | 8 | + + | N |
| Slow | 3 | TS/GLYC | 8 | − | N |

+ + +, Continuous areas of transduced cells alternated by patches of olfactory neuroepithelium containing individual cells expressing β-gal; + +, small groups and scattered cells, but no continuous areas of β-gal-positive cells; +, only scattered β-gal-positive cells; −, no expression of β-gal; OE, olfactory epithelium; T, thin; N, normal.

cavity. Infusions of virus in the right nostril of mice were performed under Hypnorm™ (diluted 1 : 10, 3 μl/g b.wt., s.c.; Janssen Pharmaceutical, Oxford, UK) and diazepam (1 μg/g i.m.; Hoffmann-La Roche, Basel, Switzerland) anaesthesia. The mice were placed on their back, a polyethylene tube (outer diameter: 0.61 mm) connected to a Hamilton microsyringe in a Harvard pump 22 was inserted into the right nostril to a depth of 7 mm and the viral vector was infused over a period of 20 min. Three different doses of defective virus particles ($1.3 \times 10^{10}$, $10^9$, $2.5 \times 10^8$ PFU) in 50 μl TS/GLYC or vehicle alone were infused (see Table 1). After infusion of the viral vector solution, the polyethylene tube was slowly removed and the mice were maintained in their position for 30 min. The mice remained under anaesthesia for at least 1.5 h.

### 2.3. Histology

Mice were perfused at various timepoints (as shown in Table 1) after viral vector infusion using 4% paraformaldehyde in 0.1 M pipes buffer (pH 7.35) containing 5 mM EGTA and 2 mM $MgCl_2$. This fixative was chosen since it effectively eliminates most endogenous β-gal activity [27] that frequently occurs in sections of rodent nervous tissues [48]. The olfactory turbinates and olfactory bulbs were dissected, post-fixed in the fixative for 2 h at 4°C and subsequently decalcified in 250 mM EDTA, 50 mM phosphate buffer, pH 7.4. After decalcification the tissue was cryoprotected by immersion in 7.5% and 15% sucrose and snap frozen in dry ice-cooled isopentane. Cryosections of 12 μm were either stained histochemically for 3 h at 37°C using 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) as a substrate for β-gal enzyme activity [50] and counterstained with haematoxylin or used for double-label immunohistochemistry to visualize β-gal and B-50/GAP-43 or β-gal and OMP in the same section. For immmunohistochemistry sections were rinsed with TBS/TX-100 (TBS containing 0.5% Triton X-100) and pre-incubated with TBS/gelatin/TX-100 (TBS containing 0.25% gelatin and 0.5% Triton X-100) for 30 min. β-gal was detected with monoclonal mouse antibodies (Sigma Immuno Chemicals; St. Louis, MO), B-50/GAP-43 was detected with

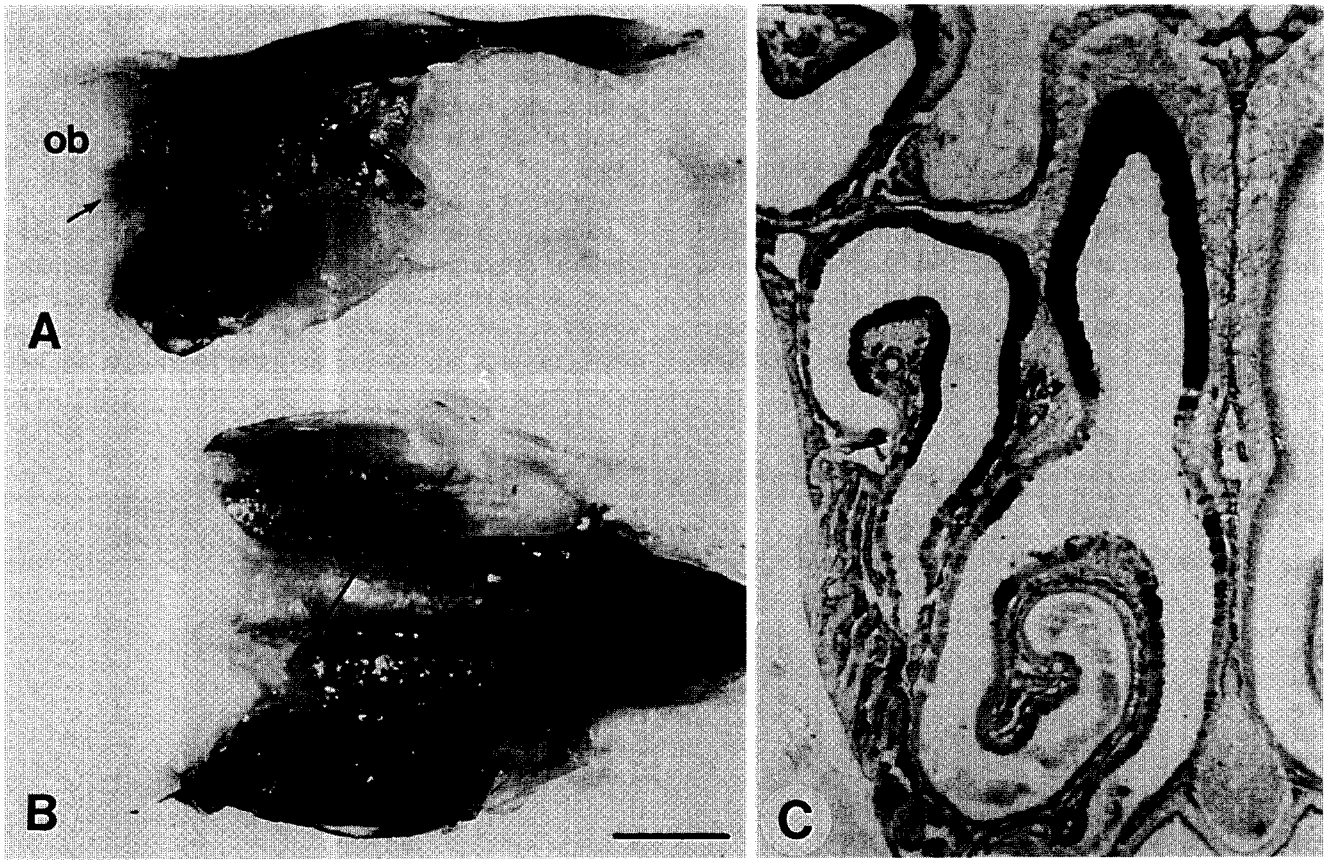


Fig. 1. Ad-LacZ directs β-gal expression to large areas of epithelium in the olfactory turbinates and septum. A,B: Low-power photographs of a whole-mount nose, stained with X-gal, 3 days after infusion of Ad-LacZ ($10^9$ PFU). Abundant β-gal expression is detected in the olfactory turbinates and septal epithelium (s) of the injected naris (right side), as well as in parts of the nerve layer (arrow) of the olfactory bulb (ob). C: Transversal section of the olfactory epithelium, stained with X-gal, 3 days after instillation of the virus ($1.3 \times 10^{10}$ PFU). Note the alternate dense and patchy β-gal staining throughout the olfactory neuroepithelium. Bar: (A) 2.2 mm; (B) 1.6 mm; (C) 500 μm.

affinity-purified polyclonal rabbit antibodies derived from antiserum #8921 (dilution 1 : 1000) [40] and OMP was detected by polyclonal goat antibodies (antiserum #255; dilution 1 : 5000) [29]. The binding of the primary antibodies was visualized with dichorotriazinylamino fluorescein (DTAF)-conjugated anti-rabbit and cy3-conjugated anti-mouse antibodies (Jackson Immuno Research Laboratories, West Grove) or biotinylated anti-mouse (Vector Laboratories, Burlingame, CA) and cy3-conjugated anti-goat antibodies (Jackson Immuno Research Lab.). The biotinylated antibodies were finally stained with fluorescein isothiocyanate isomer 1 (FITC)-conjugated streptavidin (DAKO, Glostrup, Denmark). Sections were mounted in Vectashield mounting medium (Vector Laboratories) and examined on a Zeiss confocal laser scanning microscope 410, equipped with two different lasers emitting at 488 and 543 nm, and the appropriate filters to prevent cross-talk. A stack of eight focal planes (1 μm intervals) was imaged for both fluorophores, using oil immersion objectives, whereafter one single projection was generated. A semiquantitative evaluation of β-gal expression was performed by comparing the X-gal-stained sections of all mice treated with different doses and perfused at different time points. For each mouse it was scored whether they showed continuous areas of transduced cells alternated by patches of olfactory neuroepithelium containing individual cells expressing β-gal (+ + +), small groups and scattered cells, but no continuous areas of β-gal positive cells (+ +), only scattered β-gal positive cells (+) or no expression of β-gal (−).

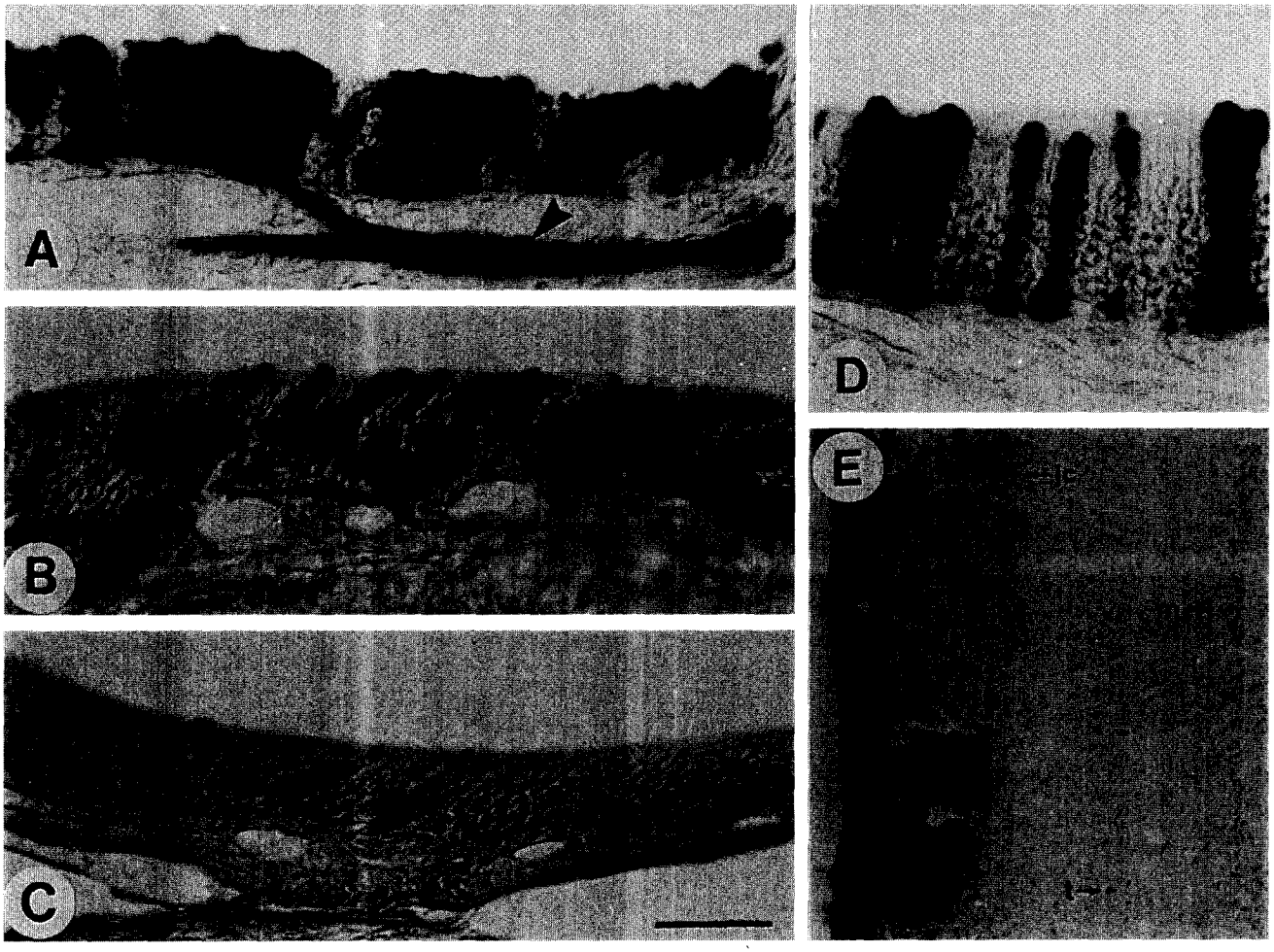


Fig. 2. β-gal is expressed by primary olfactory neurons and sustentacular cells and is transported into the axon terminals in the olfactory bulb. A–C: β-gal expression in the olfactory neuroepithelium following infusion of $10^9$ PFU. At 1 day (A) and 8 days (B) postinfusion β-gal is expressed in large numbers of olfactory neurons and sustentacular cells. Note the presence of β-gal in a nerve bundle (arrowhead) in the lamina propria in (A). At 25 days postinfusion (C) a low number of neurons expressing β-gal is still present. D: Example of β-gal expression in sustentacular cells. Note the typical apical beaker-like shape and the stalk with an expanded ending at the basement membrane. E: β-gal-positive glomeruli in the olfactory bulb at 8 days after instillation of the virus. This panel shows β-gal activity in the axonbundles of the primary olfactory neurons in the nerve layer and in the axon terminals in two dense and three patchy stained glomeruli (gl) within the olfactory bulb. Note the unlabeled second-order neurons, slightly counterstained with haematoxylin and indicated with mitral (m), tufted (t) and juxtaglomerular (jc) cells. Bar: (A–D) 50 μm; (E) 125 μm.

## 3. Results

Unilateral rapid, manual infusion of Ad-LacZ in the right nostril of mice lightly anaesthetized with ether resulted in β-gal-positive cells scattered throughout the olfactory neuroepithelium and the respiratory epithelium 3 days following viral vector application. Single cells and small groups of positive cells could be observed (data not shown).

Although significant numbers of cells expressed β-gal after a single rapid manual bolus injection of viral vector solution, the number of transduced cells did not exceed more than a few percent of the total population of cells constituting the olfactory epithelium. In an effort to improve the efficiency of adenoviral vector-mediated gene transfer the viral vector solution was infused gradually (2.5 μl/min) into the nasal cavity of fully anaesthetized mice using a microinfusion pump. After nasal infusion of three different amounts of viral vector particles (ranging from $2.5 \times 10^8$ to $1.3 \times 10^{10}$ PFU/injection per mouse) a significant improvement in the efficiency of virally transduced transgene expression became apparent (Table 1 and Fig. 1). Both neurons and sustentacular cells, identified on basis of their characteristic beaker-like shape and their typical position at the upper rim of the epithelium (Fig. 2D), were expressing the transgene, however many areas in the olfactory neuroepithelium contained only neurons expressing β-gal (Fig. 3). Infusion of $1.3 \times 10^{10}$ PFU resulted in nearly continuous areas of transduced cells alternated by patches of olfactory neuroepithelium containing individual cells expressing β-gal (Fig. 1C). Viral doses of $10^9$ PFU per mouse resulted in β-gal expression in large numbers of cells throughout the neuroepithelium (Fig. 1A,B and 2A). Application of the lowest amounts of virus ($2.5.10^8$ PFU/injection) did still result in transgene expression in a moderate number of cells. At the two lowest amounts of virus tested transgene expression persisted for 8 days (examined for $2.5 \times 10^8$ and $10^9$ PFU;

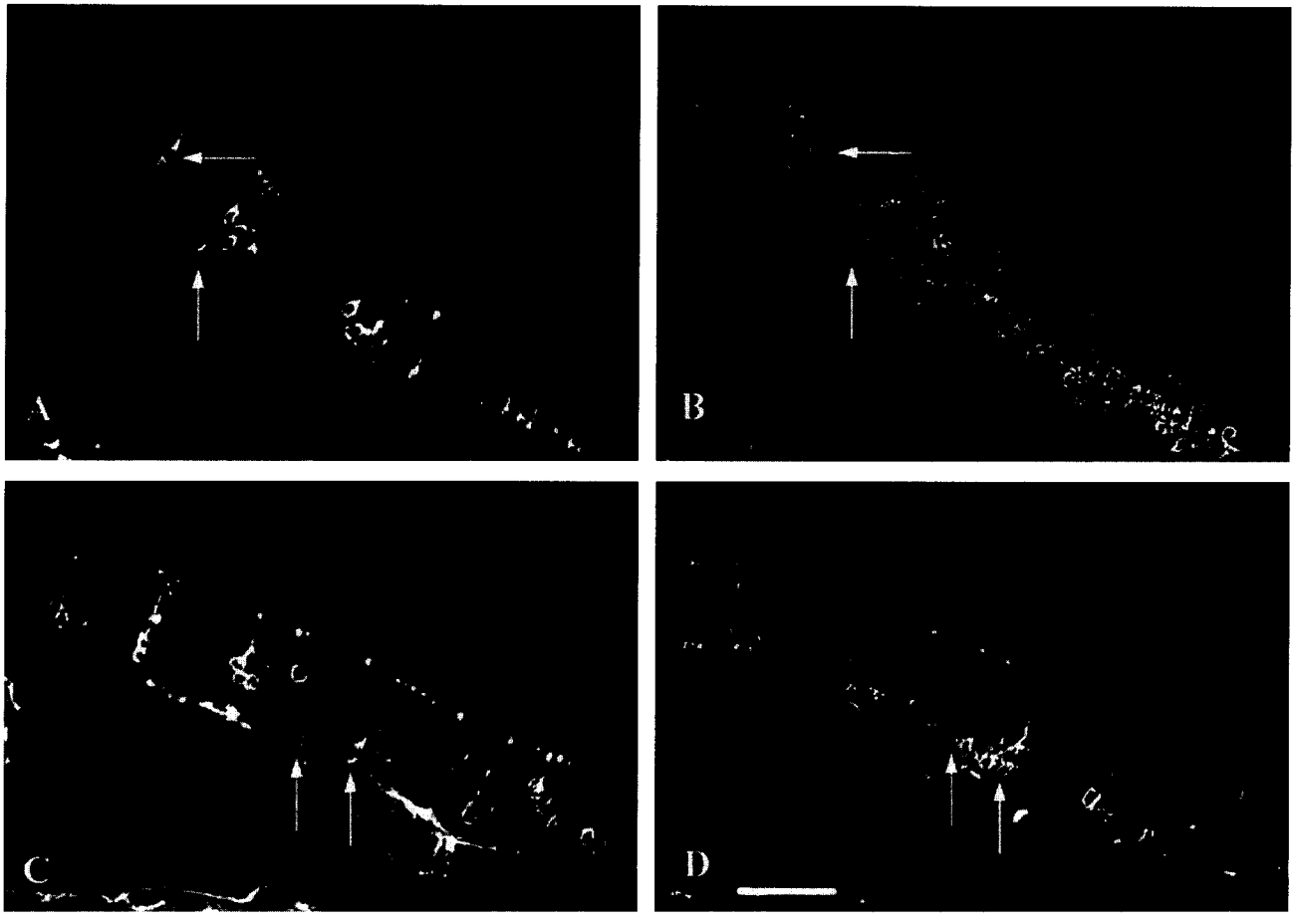


Fig. 3. Areas of olfactory neuroepithelium containing only neurons expressing β-gal, double-stained for B-50/GAP-43 and OMP. Confocal laser scanning micrographs of sections from mice, infused with Ad-LacZ ($10^9$ PFU) and perfused after 1 day (C,D) and 3 days (A,B). The olfactory epithelium sections were double-stained for β-gal (A) and OMP (B) or β-gal (C) and B-50/GAP-43 (D). A,B: Most β-gal-positive neurons expressed OMP and are located in the upper region of the olfactory neuroepithelium. Relatively low and high positioned OMP β-gal double-labelled cells are indicated by arrows. C,D: β-gal-expressing neurons in the lowest region of the epithelium express B-50/GAP-43 (arrows) indicating that immature olfactory neurons are occasionally transduced by Ad-LacZ. Bar = 50 μm.

Fig. 2B) and 12 days (examined for $10^9$ PFU). At these time points most transduced neurons had migrated into the upper three fourth of the epithelium and at 25 days following viral vector instillation only a small number of transduced cells remained (Fig. 2C). In contrast, in mice receiving the highest dose of virus ($1.3 \times 10^{10}$ PFU/injection per mouse), we observed a decrement in the number of cells expressing the transgene at 8 days postinstillation of the vector. The early drop in the number of genetically transduced cells was accompanied by a decrease in the thickness of the epithelium. This confirms previous reports on the extinction of adenoviral vector directed transgene expression in liver and lung following application of high viral vector doses and could be caused by a high multiplicity of infection (MOI) and/or an immune response elicited by the vector [6,7,11,28,49].

Starting from the first day after viral vector infusion β-gal expression was observed in numerous nerve bundles in the lamina propria (Fig. 2A), in the deeper layer of the olfactory mucosa and in the nerve layer of the olfactory bulb (Fig. 1A). At 8 and 12 days postinjection the transgene product had been transported into the nerve endings of the primary olfactory neurons in the glomeruli in the olfactory bulb (Fig. 2E). Within the glomeruli primary olfactory axons containing β-gal could be readily distinguished. β-gal expression was always restricted to sustentacular cells and dendrites or axons of primary olfactory neurons and never occurred in cells in de the lamina propria or juxtaglomerular cells and mitral cells in the olfactory bulb (Fig. 2E).

Immunohistochemical double-staining revealed that the majority of β-gal-positive neurons exhibited an OMP-positive phenotype (Fig. 3A,B). At 1 and 3 days postinfusion we also observed co-expression of B-50/GAP-43, an established marker for immature olfactory neurons, and β-gal (Fig. 3C,D). At 8, 12 and 25 days postinfusion most β-gal expressing neurons were located in the upper three-fourths of the epithelium. At these time points no neurons co-expressing β-gal and B-50/GAP-43 were observed.

## 4. Discussion

The application of replication deficient recombinant adenoviruses as vectors for gene transfer to neurons has previously been documented in vitro and in vivo [1,3,13,31]. The purpose of the present experiments was to examine the feasibility of adenoviral vector-mediated gene transfer in the olfactory neuroepithelium using a prototype recombinant adenoviral vector, Ad-LacZ. This adenoviral vector carries the *LacZ* gene under the control of a strong transcriptional activator, the CMV promoter. Here, we show that a replication deficient recombinant adenoviral vector, Ad-LacZ, efficiently directs transgene expression to neurons and sustentacular cells in the olfactory epithelium upon infusion in the nasal cavity through a nostril.

### *4.1. Efficiency of adenoviral vector-mediated gene transfer depends on viral dose and administration technique*

A rapid and manual bolus injection of a viral vector solution resulted in relatively small numbers of β-gal-positive cells scattered throughout the epithelium. The efficiency of viral vector-mediated gene transfer could be improved substantially by gradual infusion of the viral vector in the nostril. This is probably due to a better and longer contact between the viral vector solution and the epithelium. Although rapid, manual injection in one of the nostrils is by far the most convenient and quickest way to apply the vector to the mouse olfactory neuroepithelium, this procedure probably leads to clearance of most of the injected viral vector solution from the nasal cavity within minutes following application. Slow infusions of virus in mice under general hypnorm/valium anaesthesia for at least 1.5 h ensures prolonged exposure of the neuroepithelium to the viral vector solution.

At viral vector doses of $2.5 \times 10^8$ and $10^9$ PFU per mice respectively moderate and large numbers of olfactory neurons and sustentacular cells were genetically transduced. Transgene expression persisted for 8–12 days and had decreased at 25 days postinjection without any detectable signs of epithelial degeneration. The mean lifespan of the overriding majority of olfactory neurons is 30 days (reviewed by Farbman [18]). Although a small cohort of B-50/GAP-43/β-gal double labelled neurons is present at 1 and 3 days after viral instillation nearly all transduced olfactory neurons exhibited OMP expression. In the mouse OMP expression starts in primary olfactory neurons 7–8 days after they are formed from stem cells in the basal epithelium [20]. The youngest transduced OMP-positive neurons will thus have a remaining lifespan of approximately 21 days. The decrement in the number of transduced cells between 12 and 25 days is, therefore, consistent with the time course of the turnover of olfactory neurons, a natural process that occurs in the olfactory epithelium throughout adulthood [18,23,39].

The highest dose ($1.3 \times 10^{10}$ PFU/mice) of Ad-LacZ tested, initially results in large areas of densely packed β-gal-positive cells. However, after 8 days the number of β-gal-expressing cells had decreased quite dramatically and the olfactory epithelium was significantly thinner. The observed epithelial degeneration is probably caused by the high MOI, that is a high number of viral particles that is toxic for the neural cells in the epithelium [7,49], or by an immune response elicited by the adenoviral vector [6,11,28]. Relatively rapid extinction of transgene expression following the injection of high amounts of recombinant adenoviral vectors has been reported previously in monkey airway epithelium [52].

### 4.2. Ad-LacZ transduces neurons and sustentacular cells contacting the epithelial surface

Double staining for β-gal and OMP or B-50/GAP-43 at short intervals after vector application demonstrates that both mature and immature olfactory neurons are amenable to genetic manipulation with adenoviral vectors. At 8 days postinfusion no co-expression of β-gal and B-50/GAP-43 was detected. This indicates that transduced immature neurons have migrated into the upper region of the epithelium and have acquired their mature OMP-positive B-50/GAP-43 negative phenotype [20,51,57]. The relative proportion of transduced immature neurons was quite low. One explanation for this may be that immature neurons are isolated from the nasal cavity by tight junctions formed in the upper region of the epithelium between sustentacular cells and mature sensory neurons [34]. Also, immature olfactory neurons often have a dendrite with an olfactory knob that is still devoid of olfactory cilia [10,51,57]. It is not inconceivable that the cilia on the knobs of mature olfactory neurons facilitate the capture of and subsequent infection by a viral vector particle. It is remarkable that we observe areas in the epithelium containing virtually only β-gal-positive neurons and hardly any β-gal-expressing sustentacular cells (see Figs. 2 and 3). The olfactory cilia form a densely packed mat on the epithelial surface [19,35] which may locally prevent direct contact between the virus and the sustentacular cells resulting in preferential infection of mature olfactory neurons in certain regions of the epithelium. Alternatively the differential transduction of neurons and sustentacular cells could be explained by the existence of subtypes of sustentacular cells previously detected with monoclonal antibodies and by lectin-binding properties. The different subpopulations of sustentacular cells appeared to be unequally distributed throughout the olfactory epithelium [9,37].

### 4.3. Expression of β-gal is restricted to primary olfactory neurons and sustentacular cells

Previously, infection of the olfactory epithelium with wild-type viruses has been successfully performed for tracing purposes [32]. Injection of a replication competent herpes simplex virus in the nasal cavity was shown to result in transneuronal labelling of central neurons. In the present study we use an E1a/b-deleted recombinant adenovirus. The E1 deletion renders this recombinant viral vector replication incompetent [22]. The absence of β-gal enzyme activity in the target cells (mitral cells, tufted cells and juxtaglomerular cells) of primary olfactory neurons (Fig. 2E) suggests that a replication defective recombinant adenoviral vector does not spread transneuronally. A previous study, however, claimed that nasal infusion of a replication incompetent adenoviral vector results in transneuronal transduction of central neurons, i.e., mitral cells and neurons in the locus coeruleus, and cells of the choroid plexus [16]. Although replication deficient adenoviral particles can be retrogradely transported within neurons [1,3,31,21]; Hermens and Verhaagen, unpublished results), this study and previous papers have not provided evidence for passage of adenoviral vectors from one neuron to another neuron. In the study of Draghia et al. [16] 3.7% formaldehyde fixed tissue sections were incubated with X-gal for 4–12 h to detect β-gal. Under these staining conditions it is not inconceivable that the staining observed in central neurons and in the choroid plexus is due to endogenous β-gal staining [27,48]. In the present study mice were perfused with pipes buffered paraformaldehyde and stained with X-gal for 3 h, a protocol efficiently preventing background staining [27,48].

### 4.4. Adenoviral vectors are potential tools for genetic modification of the olfactory epithelium

Genetic manipulation of the olfactory epithelium has been achieved previously utilizing the transgenic mouse technique. In these studies the regulatory sequences of the *OMP* gene were used in hybrid transgenes to target the expression of a number of genes to mature olfactory neurons [12,25,30,59]. In the transgenic mouse studies the expression of the transgene started around birth and continued during adulthood, consistent with the transcriptional activity of the native *OMP* gene. Viral vector-mediated gene transfer with a recombinant vector containing the CMV promoter as a ubiquitous transcriptional activator lacks the cell-type specificity of the transgenic mouse approach using the *OMP* promoter. Nevertheless, the viral vector methodology has the advantage over the transgenic mouse technique that gene transfer can be performed in adult mice at any desired age, in an epithelium that has not been affected by the genetic manipulation during development. Cell-type specific targeting of gene expression may be achieved in the future with a recombinant adenovirus that contains the *OMP* promoter [12].

Several important applications of this technique can be envisioned. First of all, viral vector-mediated gene transfer would enable the in-vivo study of the factors that influence cell proliferation, neuronal differentiation and neuron death in the epithelium. For example, cell culture and in-situ hybridization studies have identified a number of candidate growth factors that affect different stages of differentiation of olfactory receptor neurons [14,15,24,41,46]. Adenoviral vectors encoding these growth factors would be extremely valuable tools to start to address their action in vivo. Second, adenoviral vectors could prove to be useful in the dissection of the molecular mechanisms underlying odor detection and signal transduction. Olfactory receptor genes are expressed in well-defined zones in particular populations of olfactory neurons distributed in an apparently random pattern throughout the zone [42,55]. Olfactory neurons that express a certain receptor form axonal projections in a single set of olfactory glomeruli in the bulb

[43,56]. These findings suggest that the receptor molecules may provide olfactory neurons with information that specifically instructs them to gather in a single glomerulus [43,53,56]. The olfactory receptor phenotype expressed by primary olfactory neurons appears to be independent of connections with the olfactory bulb [54]. Thus, adenovirus-mediated expression of an olfactory receptor molecule to primary olfactory neurons, that do not normally express this receptor, would allow the study of the consequences of ectopic olfactory receptor expression in an in-vivo context.

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## References

[1] Akli, S., Caillaud, C., Vigne, E., Stratford-Perricaudet, L.D., Poenaru, L., Perricaudet, M., Kahn, A. and Peschanski, M.R., Transfer of a foreign gene into the brain using adenovirus vectors, *Nature Genet.*, 3 (1993) 224–228.

[2] Anholt, R.R.H., Aebi, U. and Snyder, S.H., A partially purified preparation of isolated chemosensory cilia from the olfactory epithelium of the bullfrog, *Rana catesbeiana*, *J. Neurosci.*, 6 (1986) 1962–1969.

[3] Bajocchi, G., Feldman, S.H., Crystal, R.G. and Mastrangeli, A., Direct in vivo gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors, *Nature Genet.*, 3 (1993) 229–234.

[4] Brown, S.B. and Hara, T.J., Accumulation of chemostimulatory amino acids by a sedimentable fraction isolated from olfactory rosettes of rainbow trout (*Salmo gairdneri*), *Biochem. Biophys. Acta*, 675 (1981) 149–162.

[5] Buck, L. and Axel, R., A novel multigene family may encode odorant receptors: a molecular basis for odor recognition, *Cell*, 65 (1991) 175–187.

[6] Byrnes, A.P., Rusby, J.E., Wood, M.J.A. and Charlton, H.M., Adenovirus gene transfer causes inflammation in the brain, *Neuroscience*, 66 (1995) 1015–1024.

[7] Caillaud, C., Akli, S., Vigne, E., Koulakoff, A., Perricaudet, M., Poenaru, L., Kahn, A. and Berwald-Netter, Y., Adenoviral vector as a gene delivery system into cultured rat neuronal and glial cells, *Eur. J. Neurosci.*, 5 (1993) 1287–1291.

[8] Calof, A.L. and Chikaraishi, D.M., Analysis of neurogenesis in a mammalian neuroepithelium: proliferation in vitro, *Neuron*, 3 (1989) 115–127.

[9] Carr, V.M., Farbman, A.I., Colletti, L.M. and Morgan, J.I., Identification of a new nonneuronal cell type in rat olfactory epithelium, *Neuroscience*, 45 (1991) 433–449.

[10] Chuah, M.I., Farbman, A.I. and Menco, B.Ph.M., Influence of olfactory bulb on dendritic knob density of rat olfactory receptor neurons in vitro, *Brain Res.*, 338 (1985) 259–266.

[11] Dai, Y., Schwarz, E., Gu, D., Zhang, W.W., Sarvetnick., N. and Verma, I.M., Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression, *Proc. Natl. Acad. Sci. USA*, 92 (1995) 1401–1405.

[12] Danciger, E., Mettling, C., Vidal, M., Morris, R. and Margolis, F., Olfactory marker protein gene: its structure and olfactory neuron-specific expression in transgenic mice, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 8565–8569.

[13] Davidson, B.L., Allen, E.D., Kozarsky, K.F., Wilson, J.M. and Roessler, B.J., A model system for in vivo gene transfer into the CNS using an adenoviral vector, *Nature Genet.*, 3 (1993) 219–223.

[14] Deckner, M.L., Frisén, J., Verge, V.M.K., Hökfelt, T. and Risling, M., Localization of neurotrophin receptors in olfactory epithelium and bulb, *NeuroReport*, 5 (1993) 301–304.

[15] DeHamer, M.K., Guevara, J.L., Hannon, K., Olwin, B.B. and Calof, A.L., Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors, *Neuron*, 13 (1994) 1083–1097.

[16] Draghia, R., Caillaud, C., Manicom, R., Pavirani, A., Kahn, A. and Poenaru, L., Gene delivery into the central nervous system by nasal instillation in rats, *Gene Ther.*, 2 (1995) 418–423.

[17] Fallaux, F.J., Kranenburg, O., Cramer, S.J., Houweling, A., Van Ormondt, H., Hoeben, R.C. and Van der Eb, A.J., Characterization of 911: a new helper cell line for the titration and propagation of early-region-1-deleted adenoviral vectors, *Hum. Gene Ther.*, 7 (1996) 215–222.

[18] Farbman, A.I., Olfactory neurogenesis: genetic or environmental controls? *Trends Neurosci.*, 13 (1990) 362–365.

[19] Farbman, A.I., *Developmental and Cell Biology series, Cell Biology of Olfaction*, Cambridge University Press, Cambridge, 1992, 282 pp.

[20] Farbman, A.I. and Margolis, F.L., Olfactory marker protein during ontogeny: Immunohistochemical localization, *Dev. Biol.*, 74 (1980) 205–215.

[21] Finiels, F., Gimenez y Ribotta, M., Barkats, M., Samolyk, M.L., Robert, J.J., Privat, A., Revah, F. and Mallet, J., Specific and efficient gene transfer strategy offers new potentialities for the treatment of motor neurone diseases, *NeuroReport*, 6 (1995) 2473–2478.

[22] Graham, F.L. and Prevec, L., Manipulation of adenovirus vectors. In: E.J. Murray (Ed.), *Methods in Molecular Biology, Vol. 7, Gene Transfer and Expression Protocols*, Humana Press, Clifton, NJ, 1991, pp. 109–128.

[23] Graziadei, P.P.C. and Monti Graziadei, G.A., The olfactory system: a model for the study of neurogenesis and axon regeneration in mammals. In: C.W. Cotman (Ed.), *Neuronal Plasticity*, Raven, New York, 1978, pp. 131–153.

[24] Guthrie, K.M. and Gall, C.M., Differential expression of mRNAs for the NGF family of neurotrophic factors in the adult rat central olfactory system, *J. Comp. Neurol.*, 313 (1991) 95–102.

[25] Holtmaat, A.J.G.D., Dijkhuizen, P.A., Oestreicher, A.B., Romijn, H.J., Van der Lugt, N.M.T., Berns, A., Margolis, F.L., Gispen, W.H. and Verhaagen, J., Directed expression of the growth-associated protein B-50/GAP-43 to olfactory neurons in transgenic mice results in changes in axon morphology and extraglomerular fiber growth. *J. Neurosci.*, 15 (1995) 7953–7965.

[26] Jones, D.T. and Reed, R.R., $G_{olf}$ an olfactory neuron specific G protein involved in odorant signal transduction. *Science*, 244 (1989) 790–795.

[27] Kaplitt, M.G., Pfaus, J.G., Kleopoulos, S.P., Hanlon, B.A., Rabkin, S.D. and Pfaff, D.W., Expression of a functional foreign gene in adult mammalian brain following in vivo transfer via a herpes simplex virus type 1 defective viral vector. *Mol. Cell. Neurosci.*, 2 (1991) 320–330.

[28] Kass-Eisler, A., Falck-Pedersen, E., Elfenbein, D.H., Alvira, M., Buttrick, P.M. and Leinwand, L.A., The impact of developmental stage, route of administration and the immune system on adenovirus mediated gene transfer, *Gene Ther.*, 1 (1994) 395–402.

[29] Keller, A. and Margolis, F.L., Immunological studies of the rat olfactory marker protein. *J. Neurochem.*, 24 (1975) 1101–1106.

[30] Largent, B.L., Sosnowski, R.G. and Reed, R.R., Directed expression of an oncogene to the olfactory neuronal lineage in transgenic mice. *J. Neurosci.*, 13 (1993) 300–312.

[31] Le Gal La Salle, G., Robert, J.J., Berrard, S., Ridoux, V., Startford-Perricaudet, L.D., Perricaudet, M. and Mallet, J., An adenovirus vector for gene transfer into neurons and glia in the brain. *Science*, 259 (1993) 988–990.

[32] McLean, J.H., Shipley, M.T. and Bernstein, D.I., Golgi-like, transneuronal retrograde labelling with CNS injections of Herpes simplex virus type 1. *Brain Res. Bull.*, 22 (1989) 867–881.

[33] Margolis, F.L., A brain protein unique to the olfactory bulb, *Proc. Natl. Acad. Sci. USA*, 69 (1972) 1221–1224.

[34] Menco, B.P.M., Qualitative and quantitative freeze-fracture studies on olfactory and nasal respiratory epithelial surfaces of frog, ox, rat, and dog. III. Tight junctions, *Cell Tiss. Res.*, 211 (1980) 361–373.

[35] Menco, B.P.M., Ciliated and microvillus structures of rat olfactory and nasal respiratory epithelia. A study using ultrarapid cryo-fixation followed by freeze-substitution or freeze-etching, *Cell. Tiss. Res.*, 235 (1984) 225–241.

[36] Menco, B.P.M., Ultrastructural localization of olfactory transduction components: the G protein subunit $G_{olf\acute{o}}$ and type III adenylyl cyclase, *Neuron*, 8 (1992) 441–453.

[37] Menco, B.P.M., Lectins bind differently to cilia and microvilli of major and minor cell populations in olfactory and nasal respiratory epithelia, *Micro. Res. Tech.*, 23 (1992) 181–199.

[38] Miragall, F., Kadmon, G., Husmann, M. and Schachner, M., Expression of cell adhesion molecules in the olfactory system of the adult mouse: presence of the embryonic form of N-CAM, *Dev. Biol.*, 129 (1988) 516–531.

[39] Moulton, D.G., Cell renewal in the olfactory epithelium of the mouse. In: D.A. Denton and J.P. Coghlan (Eds.), *Olfaction and Taste, Vol. V.*, Academic Press, New York, 1975, pp. 111–114.

[40] Oestreicher, A.B., Van Dongen, C.J., Zwiers, H. and Gispen, W.H., Affinity purified anti-B50 protein antibody: interference with the function of the phosphoprotein B50 in synaptic plasma membranes, *J. Neurochem.*, 41 (1983) 331–340.

[41] Pixley, S.K., CNS glial cells support in vitro survival, division, and differentiation of dissociated olfactory neuronal progenitor cells, *Neuron*, 8 (1992) 1191–1204.

[42] Ressler, K.J., Sullivan, S.L. and Buck, L.B., A zonal organization of odorant receptor gene expression in the olfactory epithelium, *Cell*, 73 (1993) 597–609.

[43] Ressler, K.J., Sullivan, S.L. and Buck, L.B., Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb, *Cell*, 79 (1994) 1245–1255.

[44] Rhein, L.D. and Cagan, R.H., Biochemical studies of olfaction: isolation, characterization, and odorant binding activity of cilia from rainbow trout olfactory rosettes, *Proc. Natl. Acad. Sci. USA*, 77 (1980) 4412–4416.

[45] Rogers, K.E., Dasgupta, P., Gubler, U., Grillo, M., Khew-Goodall, Y.S. and Margolis, F.L., Molecular cloning and sequencing of a cDNA for olfactory marker protein, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 5218–5222.

[46] Ronnett, G.V., Hester L.D. and Snyder, S.H., Primary culture of neonatal rat olfactory neurons, *J. Neurosci.*, 11 (1991) 1243–1255.

[47] Ronnett, G.V., Cho, H., Hester, L.D., Wood, S.F. and Snyder, S.H., Odorants differentially enhance phosphoinositide turnover and adenylyl cyclase in olfactory receptor neuronal cultures, *J. Neurosci.*, 13 (1993) 1751–1758.

[48] Rosenberg, W.S., Breakefield, X.O., DeAntonio, C. and Isacson, O., Authentic and artifactual detection of the *E. coli* LacZ gene product in the rat brain by histochemical methods, *Mol. Brain Res.*, 16 (1992) 311–315.

[49] Sabaté, O., Horellou, P., Vigne, E., Colin, P., Perricaudet, M., Buc-Caron, M.H. and Mallet, J., Transplantation to the rat brain of human neural progenitors that were genetically modified using adenoviruses, *Nature Genet.*, 9 (1995) 256–260.

[50] Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning. a Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, New York, 1989.

[51] Schwob, J.E., Mieleszko Szumowski, K.E. and Stasky, A.A., Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival, *J. Neurosci.*, 12 (1992) 3896–3919.

[52] Simon, R.H., Engelhardt, J.F., Yang, Y., Zepeda, M., Weber-Pendleton, S., Grossman, M. and Wilson, J.M., Adenovirus-mediated transfer of the *CFTR* gene to lung of nonhuman primates: toxity study, *Hum. Gene Ther.*, 4 (1993) 771–780.

[53] Singer, M.S., Shepherd, G.M. and Greer, C.A., Olfactory receptors guide axons, *Nature*, 377 (1995) 19–20.

[54] Sullivan, S.L., Bohm, S., Ressler, K.J., Horowithz, L.F. and Buck, L.B., Target-independent pattern specification in the olfactory epithelium, *Neuron*, 15 (1995) 779–789.

[55] Vassar, R., Ngai, J. and Axel, R., Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium, *Cell*, 74 (1993) 309–318.

[56] Vassar, R., Chao, S.K., Sitcheran, R., Nuñez, J.M., Vosshall, L.B. and Axel, R., Topographic organization of sensory projections to the olfactory bulb, *Cell*, 79 (1994) 981–991.

[57] Verhaagen, J., Oestreicher, A.B., Gispen, W.H. and Margolis, F.L., The expression of the growth associated protein B50/GAP43 in the olfactory system of neonatal and adult rats, *J. Neurosci.*, 9 (1989) 683–691.

[58] Viereck, C., Tucker, R.P. and Matus, A., The adult rat olfactory system expresses microtubule-associated proteins found in the developing brain, *J. Neurosci.*, 9 (1989) 3547–3557.

[59] Walters, E., Grillo, M., Tarozzo, G., Stein-Izsak, C., Corbin, J., Bocchiaro, C. and Margolis, F.L., Proximal regions of the Olfactory Marker Protein gene promoter direct olfactory neuron-specific expression in transgenic mice, *J. Neurosci. Res.*, 43 (1996) 146–160.