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Mild surfection of neural cells, especially motoneurons, in primary culture and cell lines

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Abstract

Of all cell types, motoneurons (MNs), are possibly the most difficult to maintain in culture, since their development and survival is conditioned by many factors that are still in the course of identification. This may also be the reason why they are difficult to transfect. We succeed to transfect these fragile cells with lipoplex [DOTAP:PC (10:1)-pGFP]-precoated coverslips. Here, we report that this original method, also termed 'surfection' does not perturbate MN development and survival while giving important transfection yield (15%). Lipofectamine™ 2000 and other well-known auxiliary lipids (DOPE, Chol) give lower surfection yields.

The use of (DOTAP:PC)-based lipid vector also can be extended to several neural and non-neural cell lines with appreciable transfection yield such as a glial cell line (GCL) derived from rat spinal cord (65%), HeLa S3 (60%), COS-7 (30%) and HEK 293 cells (20%).

The efficiency of DOTAP:PC (10:1) and Lipofectamine™ 2000 vectors in our surfection method are compared on standard HeLa S3 cell lines. Lipofectamine™ 2000 (72%) is slightly better than DOTAP:PC (10:1) (60%). However, the surfection method improved the efficiency of Lipofectamine™ 2000 itself (72%) as compared to the classical (62%) approach.

In summary we have developed an original standard surfection protocol for both MN primary cultures and cell lines, thus simplifying laboratory practice; moreover, Lipofectamine™ 2000 used in this surfection method is more efficient for the cell lines than the manufacturer-recommended method. We emphasize that our method particularly spares fragile cells like MNs from injure and therefore, might be applied to other fragile cell type in primary cultures.

Keywords: Cationic liposome; Surfection; Primary motoneurons; Cortical neurons; Glial cells; Lipoplex-coated coverslip; DOTAP; DOPE; Cholesterol; GFP

Introduction

At the present time, several non viral techniques are commercially available for transfecting a foreign gene into a neuronal cell in primary culture, such as the nucleofection system based on electroporation (Neuhuber and Daniels, 2003) or those requiring the new generation of well-known monovalent (NeuroPorter™; Sigma-Aldrich, USA) or multivalent lipids (Lipofectamine™ 2000; Invitrogen, USA). Although attention is often focused on transfection efficiency, the goal of the study should not be forgotten. For example, if the objective is to investigate the toxic effect of a foreign protein in a given cell type, it is preferable to spare the cells by using a minimally aggressive method even at the cost of reduced yield. Otherwise, results would be biased from the outset. Therefore it would be inappropriate to use for example the nucleofector electroporation method for studying a mutated Cu/Zn-superoxide dismutase (SOD1; 2% of Amyotrophic Lateral Sclerosis origin), a

Abbreviations: Chol, cholesterol; DOPE, dioleylphosphatidylethanolamine; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium lipid; FCS, fetal calf serum; GFP, green fluorescent protein; HBSP, Hanks' buffered saline plus supplements; PBS, phosphate-buffered saline; PC, L-α-phosphatidylcholine; TRITC, tetramethyl rhodamine isothiocyanate.

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death-associated protein (DAXX, Fas-mediated apoptosis) or a mutated androgen receptor ($_m$AR, Kennedy's disease) in primary cultured motoneurons (MNs). Indeed, even a minimal electric shock might provoke the death of MNs in primary culture. Moreover, development and survival of cultured MNs derived from the ventral spinal cord of rodent embryos (E15) are sensitive to the slightest fluctuation of culture conditions such as the physical disturbance of cell environment or modification of the medium composition (constituents, serum concentration, presence of antibiotics and growth factors) (Arce et al., 1999; Ricart et al., 2006). The synthetic lipid vectors cited above may not be very aggressive to cultured MNs, but the technique by itself has several drawbacks: (i) in current lipid vector methods, cells are initially seeded and then the lipoplex (Even-Chen and Barenholz, 2000; Hofland et al., 1996) or the polyplex (multicationic lipid+plasmid) (Thomas and Klibanov, 2002; Tang et al., 2003) is added to the culture medium. Thus, liquid movements are generated by the multiple media changes during the transfection step; (ii) the use of special buffers like Opti-MEM®-I (Gibco Brl, USA) diminishes MN survival and (iii) antibiotics and concentrated serum, two factors indispensable for MN survival have to be present at cell seeding and all along the cell culture, but are detrimental to efficiency of transfection.

Recently, a novel approach of the transfection process, which consists in coating the coverslip with the lipoplex before cell seeding, has been described and proved reliable (Shea et al., 1999; Zheng et al., 2000; Ziauddin and Sabatini, 2001; Chang et al., 2004). However, to our knowledge this single-step matrix-surface-mediated transfection method (also termed surfection or reverse transfection) has been applied only to cell lines that divide and develop easily and that are easy to maintain in culture. We adapted this method for neuronal cells in primary culture, especially motoneurons, for the following reasons: (i) the classical polylysine/laminin network used as adherent substrate for motoneurons is also valid for trapping lipoplex added later instead of biocompatible biopolymers such as poly (lactic-co-glycolic)acid (PLGA) (Shea et al., 1999; Zheng et al., 2000), collagen (Bielinska et al., 2000) or gelatin (Ziauddin and Sabatini, 2001); (ii) in order to reduce injury during cell transfection, we use natural and/or well-known fusogenic lipids as liposome constituents.

Our original approach allowed to transfect, with a good yield, very fragile cells such as MNs derived from E15 Embryos and E17 rat cortical neurons but also standard cell lines.

We have found that transfected MNs are suitable for studying cellular events. The same method of transfection is also valid for spinal cord glial lines or other well established cell lines; thus, standardizing the transfection method.

Materials and methods

Plasmids

The green fluorescent protein (GFP) plasmid (pGFP) derived from the jellyfish *Aequorea victoria* (Chalfie et al., 1994; Heim et al., 1995) the wild-type androgen receptor AR fused to GFP plasmid (pGFP−$_{wt}$AR; normal receptor with 21 CAG) and the mutated receptor fused to GFP plasmid (pGFP−$_m$AR; altered receptor with 51 CAG) were gifts from Dr. S. Lumbroso's research group (U439 INSERM). Plasmid DNA was amplified and purified by the Qiagen Plasmid Maxi Kit (Qiagen, USA).

Lipid and detergent stock solutions

Lipids and detergent were dissolved previously in chloroform in a volume shown in Table 1. Samples were placed into sturdy vial with a conical interior bottom (wheaton v-vial; Electron Microscopy Sciences; USA) and stored at −20°C in a nitrogen atmosphere and for up to 2 weeks.

Lipid formulations

The lipid composition of liposomes was *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium (DOTAP; Sigma, France) combined with L-α-phosphatidylcholine (PC; Sigma, France), cholesterol (Chol; Sigma, France) or dioleylphosphatidylethanolamine (DOPE; Fluka, Switzerland). We tested different types of liposomes listed in Table 2.

Liposomes

Liposomes were obtained by the extrusion method according to Felgner et al. (1987) and Maruyama et al. (1990) with some modifications. Volumes of DOTAP, PC (Chol or DOPE) and *n*-hexyl-β-D-glucopyranoside (Sigma; France) as specified in Table 1 were taken from their stock solution, and mixed vigorously in a conical tube and evaporated in a culture incubator overnight. The viscous residue thus formed was redissolved in 200 µl of Hanks' buffered saline plus supplements (HBSP) containing in mM: Na_2HPO_4 0.75 (Merck, Germany), KCl 5 (Prolabo, France), NaCl 140 (Prolabo, France), glucose 6 (Merck, Germany) and HEPES 25 (Sigma, Germany). The solution was shaken and dialysed against HBSP for 10 h (spectra/por® microdialyzer, Spectrum®, USA). The molecular weight cut-off of the dialysis membrane was 10 kDa (spectra/por® CE dialysis membrane). Before dialysis, the buffered solution was placed in a shaker for 1 h to make vacuum

Table 1
Lipid and detergent stock solutions

Stock solution in chloroform		DOTAP 3 mg/ml	PC 3.24 mg/ml	DOPE 10 mg/ml	Chol 1 mg/ml	Detergent 1 mg/385 µl
Volume (µl) to take for	DOTAP: PC 10:1	100	10			50
	DOTAP: PC 1:1	100	100			93.6
	DOTAP: DOPE 1:1	100		32		93.6
	DOTAP: Chol 1:1	100			166.3	93.6

Table 2
Lipid formulations

	Liposomes			
	DOTAP:PC		DOTAP:DOPE	DOTAP:Chol
Molar ratio	10:1	1:1	1:1	1:1
µmol for 1 ml liposome	0.43:0.043	0.43:0.43	0.43:0.43	0.43:0.43
Detergent (µmol)	0.43	0.92	0.92	0.92

degassing easier. After dialysis, the volume of the liposome suspension obtained was adjusted to 1 ml using HBSP buffer.

Lipoplex

Plasmid DNA and liposome suspensions (in proportions cited in Table 2) were each diluted in an equal volume of HBSP. After mixing gently three times for 5 min, the two solutions were mixed (final volume 200 µl) and incubated for 20 min at room temperature before coverslip coating.

R parameter (R = lipid charge$^{(+)}$/plasmid DNA charge$^{(-)}$)

Two values for R were tested: $R=2$ and 4, i.e., a mixture of 40 and 80 µl of DOTAP:PC (10:1) liposome with 0.16×10^{-7} positive charges or 0.32×10^{-7} positive charges, respectively, and 3 µl (1 µg/µl) of pGFP with 6.7 kb (8.64×10^{-9} negative charges).

Transfection process

Transfection was achieved by coating the coverslips with the solution of lipoplex before cell seeding. For this, sterile coverslips (F 22 mm diameter, CML, France) were incubated beforehand with 0.002% (w/v) poly-D-lysine (MW > 300,000, Sigma, France) overnight, then with 0.0003% (w/v) laminin (Sigma, France) for 4 h and finally with lipoplex suspension overnight at 35°C in a CO_2 incubator. Transient transfection experiments were carried out for 24 to 72 h, before fixing the cells.

For classical transfection, cells were seeded and transfected according Invitrogen™ life technologies procedures: volume of culture medium (500 µl), quantity of pDNA (0.8 µg), volume of Lipofectamine™ 2000 (2 µl) and Opti-MEM®-I reduced Serum Medium as solution to dilute the transfection agent.

During the comparative surfection/classical transfection tests: (i) we did not remove the transfection solution with the classical method; (ii) we have synchronized the start-time of transfection (T_0): cells for classical transfection were seeded 24 h sooner than those assigned for surfection. At T_0, this pool of cells was incubated with pGFP complexed with Lipofectamine™ 2000 and a second pool of cells was seeded on two types of precoated dishes: Lipofectamine™ 2000 or DOTAP:PC (10:1).

Cell culture

Human embryonic kidney cell line (HEK 293) was kindly provided by Sanofi-Synthelabo (Labège, France). Dr Lumbroso (Hôpital Caremeau, CHU Nîmes, France) provided HeLa S3 epithelioid carcinoma cells (HeLa S3) and SV40-transformed kidney fibroblasts from the African green monkey (COS-7).

Glial cells

Rat spinal cord glial cell lines were derived from MN cultures (see below) as previously described (Rakotoarivelo et al., 2004). Neuronal cells were left to necrose and the confluent remaining cells were harvested by 250 µl of a 0.05% trypsin solution (EC. 3.4.21.4. from bovine pancreas, type IIIS, Sigma, France) containing 0.02% EDTA (Merck, Germany), 0.80% NaCl (Prolabo, France), 0.04% KCl (Prolabo, France), 0.06% sodium bicarbonate (Merck, Germany) and 0.1% glucose (Merck, Germany) for 4 min at 35°C. Cells were collected and mechanically suspended in culture medium I (CMI) which consisted of 70% MEM: 30% Hanks' Balanced Salt Solution (HBSS, Invitrogen, USA) (v/v), adjusted with sodium bicarbonate to 0.17%, 5 µg/ml Ciflox (Ciprofloxacin, Bayer, Germany), 2.5 µg/ml Fungizone (Invitrogen, USA) and 10% Foetal Calf Serum (FCS, Invitrogen, USA) and pelleted at 65 g for 8 min. Cells were resuspended again in CMI and seeded in 25 cm^2 ventilated culture flasks (Falcon, USA) and kept (CO_2 incubator) in a 5% CO_2 humid atmosphere (Ultrapure water: MilliQ water filtered with 0.22 µm Millipore filters) at 35°C. Growth medium was changed once a week.

Motoneurons

Motoneuron cultures were obtained as described previously (Rakotoarivelo et al., 2004). Briefly, pregnant Sprague-Dawley rats (15th day of gestation) were anesthetized and the uterine string was removed. Embryos were separated in two halves following a line starting from the mesencephalic curve to the sacrum. The side containing the spinal cord was cleared of surrounding tissue and spinal cords were cut at the level of the rhombencephalic curve. The ventral halves were placed in 1 ml chilled CMII which consisted of CMI supplied with 0.80% NaCl, 0.12% KCl, 0.60% glucose and 0.05% sodium pyruvate (Sigma, France).

Ventral spinal cord fragments (0.5 ml) were gently dissociated at room temperature in culture medium III (CMIII), consisting of CMII to which 20% FCS, 0.10% bovine serum albumin (BSA, USA) (fraction V, culture-tested, Sigma), insulin (5 µg/ml, Sigma, USA), putrescine (160 µg/ml, Sigma, USA), conalbumine (100 µg/ml, Sigma, USA), sodium selenite (5 ng/ml, Sigma, USA) and progesterone (6.28 ng/ml, Sigma, USA) were added. After complete dissociation, 1 ml cellular material was purified by two centrifugation steps through nicodenz (Sigma, USA) and BSA cushions according to Camu and Henderson (1992) with the modifications described in our previous work (Rakotoarivelo et al., 2004). Motoneurons were then seeded in CMIII with muscle extract added to a final concentration of 8% (v/v) on coated polylysine/laminin coverslips.

Cortical neurons

Cerebral cortical tissue obtained according to Petite and Calvet (1995) with some modifications from E17 rat embryos. After transfection of the corpus callosum the cerebral hemispheres were gently pressed apart, separated from the subcortical structures and then mechanically dissociated in

phosphate-buffered saline (PBS; Sigma, France) solution. Cells were seeded in a culture medium consisted in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% FCS, 100 U/ml penicillin (Sigma, France) and 100 μg/l streptomycin (Sigma, France).

Animals were housed under a controlled light cycle (12 h light/12 h darkness) and were given standard food and water freely (accreditation number of animal house of Institut des Neurosciences de Montpellier: A3417231).

Standard cell lines

Cell lines were grown under standard cell culture conditions (37°C, 5% CO_2) in DMEM solution supplemented with 10% FCS serum or 10% desteroided fetal calf serum (gift from Dr Lumbroso) (Sigma's protocol according Green and Leake, 1987) and 5 μg/ml ciflox.

Cell density

All cell types were studied either at a low confluence [between 5000 and 10,000 cells/2.2 cm diameter culture dish (Falcon, USA)] or 3/4 confluence (between 30,000 and 40,000 cells/coverslip) except for the cortical neurons which were seeded at 100,000 cells/coverslip.

Immunocytochemistry

Fixation

Cell lines were fixed with 4% paraformaldehyde (Merck, Germany) in PBS for 45 min at room temperature. Neuronal cells were fixed with either paraformaldehyde or glutaraldehyde (Merck, Germany) as described previously (Rakotoarivelo et al., 2004).

Neuronal staining

Fixed cells were permeabilized for 1 h with 2% BSA and 0.4% (v/v) Triton X-100 (Sigma, USA) in PBS and then incubated overnight in PBS at 4°C with rabbit polyclonal anti-peripherin (Chemicon, USA) or mouse monoclonal anti-β-tubulin III primary antibodies (Sigma, Germany) diluted 1/1000 or 1/500 in 0.1% Triton X-100 and 2% BSA to identify MN and cortical neurons, respectively. Cells were then rinsed three times with PBS for 5 min and incubated for 1 h at room temperature with tetramethyl rhodamine isothiocyanate (TRITC)-linked secondary antibodies (Sigma; France) diluted 1/2000 and 1/1000, respectively.

In some experiments for MNs, the GFP signal was amplified by rabbit polyclonal anti-GFP primary antibodies (Molecular probes, USA) diluted 1/1000 and either with goat TRITC- (Sigma; France) or sheep FITC- (Sigma; France) linked secondary antibodies diluted 1/2000 and 1/200, respectively.

Nuclear staining

After washing with PBS, nuclei were stained with the DNA-intercalating dye bisbenzimide (Hoechst 33342; 1 μg/ml in PBS; Sigma, USA) for 15 min at room temperature. Cells were rinsed again as above.

Cell viability assay

Cell viability was visualized by mitochondrial succinic dehydrogenase reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) into insoluble purple formazan product according to Liu et al. (1997) and quantified by Trypan blue (Sigma, USA) exclusion assays (Freshney, 1987).

Fluorescence

Coverslips were mounted using Vectashield fluorescence mounting medium (Interchim, France) and observed by epi-fluorescence microscopy (U-RFL-T type; Olympus, Japan) coupled to a camera (Photonics Science, UK) and to image-processing software (Image Pro-plus, USA).

GFP expression per dish

GFP expression was measured in two ways: either by calculating the GFP fluorescence/Hoechst fluorescence ratio per dish or the GFP fluorescence only per dish. We used the first method to measure the effect of cell density on transfection rate and the second method for the effect of the neutral lipid constituent on transfection efficiency.

For this, after 24 h of transfection, cells were rinsed three times and harvested mechanically with 1 ml of PBS solution and fluorescence was measured with a VersaFluor™ Fluorometer (BioRad, France). Excitation filters 360/40 and 490/10 were used for Hoechst and GFP, respectively. Emission filters 460/10 and 510/10 for Hoechst 33342 and GFP, respectively. Fluorescence in the figures is in arbitrary units (a.u).

Statistics

Experiments were carried out three times. Statistic analysis was carried out using Student's *t*-test if two sets of data were compared. ANOVA test was used if more than two sets were compared. In the text, 'degrees of freedom' is abbreviated '*df*'. A probability (*P*) of less than 0.05 was considered significant.

Results

R parameter assessment for DOTAP:PC (10:1)/pGFP

The lipid vector was composed of DOTAP:PC (10:1). For the determination of the optimal value for the lipid/DNA charge ratio *R* we tested two frequently used values: $R=2$ and 4. Before cell seeding, the coverslips were coated with polylysine, then with laminine and finally coated with pGFP-based lipoplex. After 72 h of GCL transfection, cell counting indicated: 52.87±3.21% and 63.54±3.91% for $R=2$ and $R=4$, respectively (Table 3). Student's *t*-test analysis is significant: $t=2.167$, $P<0.05$. We found essentially the same result (Table 3) for MNs. The surfection yields after 72 h were: 10.04±1.92% and 15.21±2.83% for $R=2$ and $R=4$, respectively. Student's *t*-test analysis is: $t=1.556$, ns.

Thus, The $R=4$ value was retained for further experiments.

Table 3
R parameter

	Lipid/DNA charge ratio	Surfection yield (%)
GCL	$R=2$	52.87 ± 3.21 [a]
	$R=4$	63.54 ± 3.91 [a]
MNs	$R=2$	10.04 ± 1.92 [b]
	$R=4$	15.21 ± 2.83 [b]

[a] $t=2.167$ and $P<0.05$.
[b] $t=1.556$; ns.

Immunocharacterization of GFP-positive neurons in primary culture

We used the same method of transfection for MNs and cortical neurons in primary cultures. Hence, we kept the polylysine/laminin network as adherent molecules and used DOTAP:PC (10:1)/pGFP as lipoplex. After 48 h of transfection, cells were identified by specific antibodies: anti-intermediate filament protein, peripherin, as MN marker and anti-β-tubulin III as cortical neuron marker.

In MN cultures, we observed some double-labelled cells: GFP (green fluorescent protein)- and peripherin (red TRITC fluorescence)-positive MNs (Fig. 1A). We confirmed the identity of the transiently expressed protein using an antibody directed against GFP protein (red TRITC fluorescence). GFP-positive MNs co-stained with the anti-GFP antibodies are shown in Fig. 1B.

GFP- and type III β-tubulin-positive (red TRITC fluorescence) double-labelled cells in cortical neuron cultures are shown in Fig. 1C.

Surfection of MNs in primary culture

DOTAP:PC (10:1) versus DOTAP:DOPE (1:1) or DOTAP:Chol (1:1)

We compared the transfection efficiency of DOTAP:PC (10:1) with two widely used standard liposomes DOTAP:DOPE (1:1) and DOTAP:Chol (1:1). MNs were seeded as detailed above and treated with pGFP-based lipoplex for 48 h. Results are (mean±SEM): 6.06±0.21; 25.90±1.12; 19.70±0.84 and 18.10±0.64 for: control cells (auto-fluorescence), DOTAP:PC

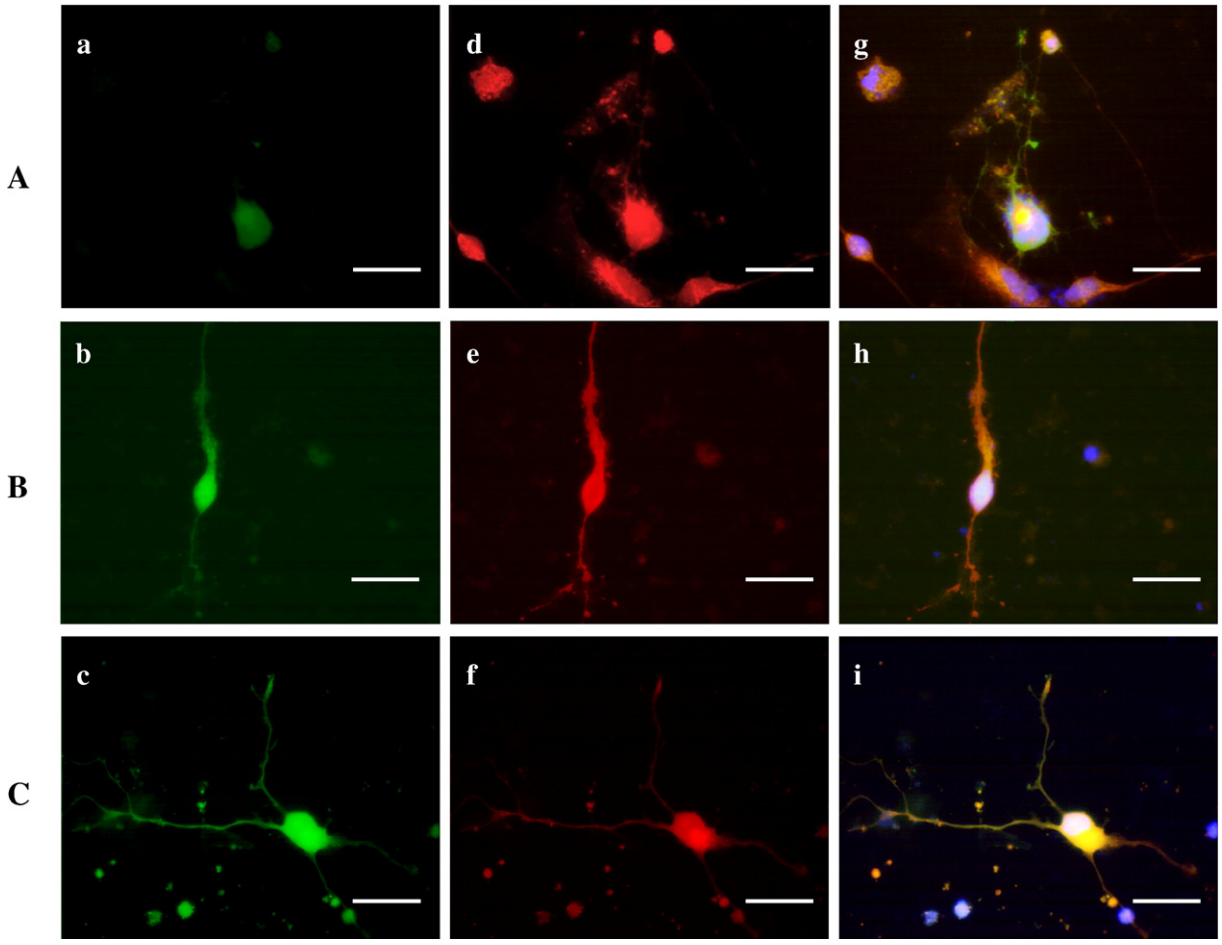

Fig. 1. Fluorescence microscope images of GFP-positive MNs (A; B) and a cortical neuron (C) seeded on coverslips coated with lipoplex based on DOTAP:PC 10:1 and pGFP. After 2 days of culture, and hence transfection, cultured motoneurons and cortical neurons already express specific markers: peripherin (d) and β-tubulin III (f) (red), respectively. Some of them also express the transient protein GFP (a, b, c) (green). Transfected MNs express GFP that is recognised by antibodies directed to GFP (TRITC) (e). In all cases, triple labeling with Hoechst 33342 has been carried out (g, h, i) (blue). Scale bars represent 25 μm.

(10:1), DOTAP:DOPE (1:1) and DOTAP:Chol (1:1), respectively (Fig. 2). ANOVA analysis ($F=128$; $df=3$) reveals significant differences for control versus DOTAP:PC (10:1) ($^{€}P<0.001$), control versus DOTAP:DOPE (1:1) ($^{£}P<0.001$), control versus DOTAP:Chol (1:1) ($^{§}P<0.001$), DOTAP:PC (10:1) versus DOTAP:DOPE (1:1) ($^{*}P<0.001$) and DOTAP:PC (10:1) versus DOTAP:Chol (1:1) ($^{\$}P<0.001$).

These data demonstrate that DOTAP:PC (10:1) has a 24% larger transfection efficiency than DOTAP:DOPE (1:1) and a 30% larger transfection efficiency than DOTAP:Chol (1:1). Thus, the DOTAP:PC (10:1) seems to be the most efficient lipid vector for MN transfection.

We also investigated the morphology of control and transfected cells by phase contrast microscopy. Pictures were taken just before fluorescence evaluation. Cells treated with pGFP complexed to either DOTAP:PC (10:1) or DOTAP:DOPE (1:1) extended neurites similar to those of (a) for control cells. In contrast, most cells treated with the lipoplex containing DOTAP:Chol (1:1) (Fig. 2D) did not have visible neurites.

With regard to cells transfected with pGFP complexed with DOTAP:PC (10:1) or DOTAP:DOPE (1:1), all the cells displayed formazan formation 48 h after transfection (Fig. 3A) thus confirming that the proposed method does not alter cell viability. Moreover, cell viability was quantified by Trypan blue exclusion assays. Results (mean±SEM) of cell counting based on 300 cells after 72 h transfection indicate that: 95.0±2.4%; 93.7±2.6% and 89.7±2.1% of cells are alive for: DOTAP:PC (10:1), DOTAP:DOPE (1:1) and

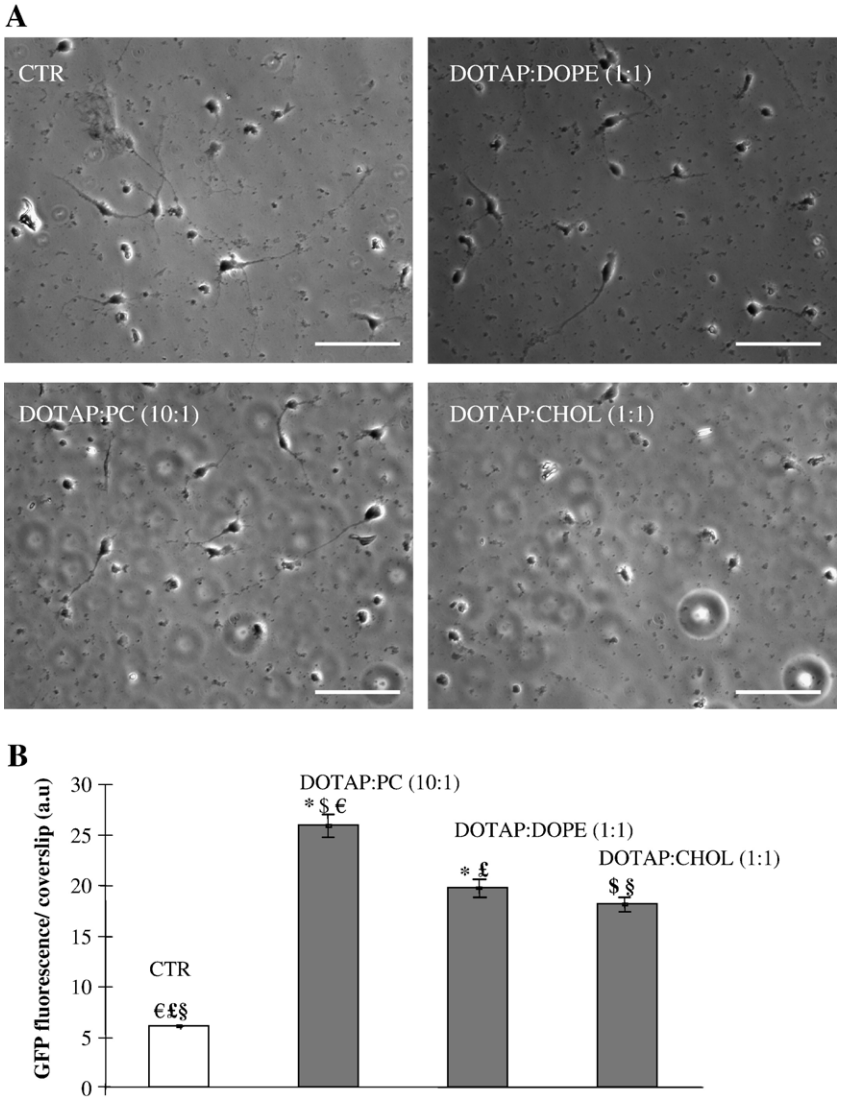

Fig. 2. Comparative analysis of the influence of auxiliary lipids (PC, DOPE, Chol) on the efficiency of the lipoplex-coated dish method by measurement of GFP fluorescence in primary MNs with corresponding phase-contrast micrographs. After 48 h of transfection with pGFP combined with different lipoplexes, living cells were initially examined directly by phase-contrast microscopy. (A) Neurites of cells growing on DOTAP:PC (10:1) and DOTAP:DOPE (1:1) have a similar morphology as those of control cells while most cells growing on DOTAP:Chol (1:1) do not have visible neurites (scale bars represent 100 μm). Then, cells were harvested mechanically with 1 ml of PBS solution and fluorescence was measured. (B) Histograms reflect GFP- (or auto-) fluorescence/coverslip: control cells (white bar); transfected cells (grey bars). Data are means±SEM obtained from nine coverslips of three independent experiments. ANOVA analysis ($F=128$; $df=3$) reveals significant differences for control versus DOTAP:PC (10:1) ($^{€}P<0.001$), control versus DOTAP:DOPE (1:1) ($^{£}P<0.001$), control versus DOTAP:Chol (1:1) ($^{§}P<0.001$), DOTAP:PC (10:1) versus DOTAP:DOPE (1:1) ($^{*}P<0.001$) and DOTAP:PC (10:1) versus DOTAP:Chol (1:1) ($^{\$}P<0.001$).

control, respectively (Fig. 3B). ANOVA analysis ($F=1.603$; $df=2$) reveals no significant difference between parameters. As positive control, we treated MNs with 0.3 μM H_2O_2 in PBS for 2 h before Trypan blue incubation. All nuclei are colored blue.

Independent experiments were undertaken to visualize GFP-positive cells. Results in Fig. 4 represent the fluorescence micrographs of cells transfected with either DOTAP:DOPE (1:1) (Fig. 4A) or DOTAP:PC (10:1) (Fig. 4B).

DOTAP:PC (10:1) versus Lipofectamine™ 2000

Lipofectamine™ 2000 was used as lipid coating for the MN surfection and compared with DOTAP:PC (10:1). Cell counting of fluorescent GFP-positive MNs after 48 h indicate: 14.91±4.61% of transfected cells for DOTAP:PC (10:1) versus 4.61± 1.22% for Lipofectamine™ 2000 (Fig. 5A). Student's *t*-test analysis gives $t=5.965$ ($P<0.001$).

Effect of cell density on DOTAP:PC (10:1) surfection

MNs were seeded at 10,000, 20,000 and 40,000 cells per coverslip. Cell counting of fluorescent GFP-positive MNs after 72 h indicates no significant differences.

In order to verify if our method works with cell lines, we have carried out the following experiments.

Surfection of cell lines versus MNs

DOTAP:PC (10:1) on several cell lines and MNs

Cells were seeded at low density. Cell counting after 72 h transfection indicated 15.21±2.82%, 64.53±0.68%, 60.18± 1.36%, 30.33±1.27% and 20.13±1.70% (triplicate measures) of transfected MNs, GCL, HeLa S3, COS-7 and HEK 293 cells, respectively (Fig. 6A). ANOVA analysis ($F=187.5$; $df=4$) reveals significant differences between: MNs versus GCL, HeLa S3 and COS-7 ($, *, #), GCL versus HeLa S3, COS-7 and HEK 293 (€, §), HeLa S3 versus COS-7 and HEK 293 (£, &) and COS-7 versus HEK 293 (†) ($^{\$, *, \#, €, §, £, \&, †}P<0.05$).

Effect of cell density on DOTAP:PC (10:1) transfection

In order to estimate the influence of cell density on transfection, we tested two conditions on GCL: low and 3/4 confluence. After 24 h of transfection, culture dishes were rinsed once with PBS buffer. Then, cells were mechanically collected with 1 ml PBS and placed into a plastic cell for

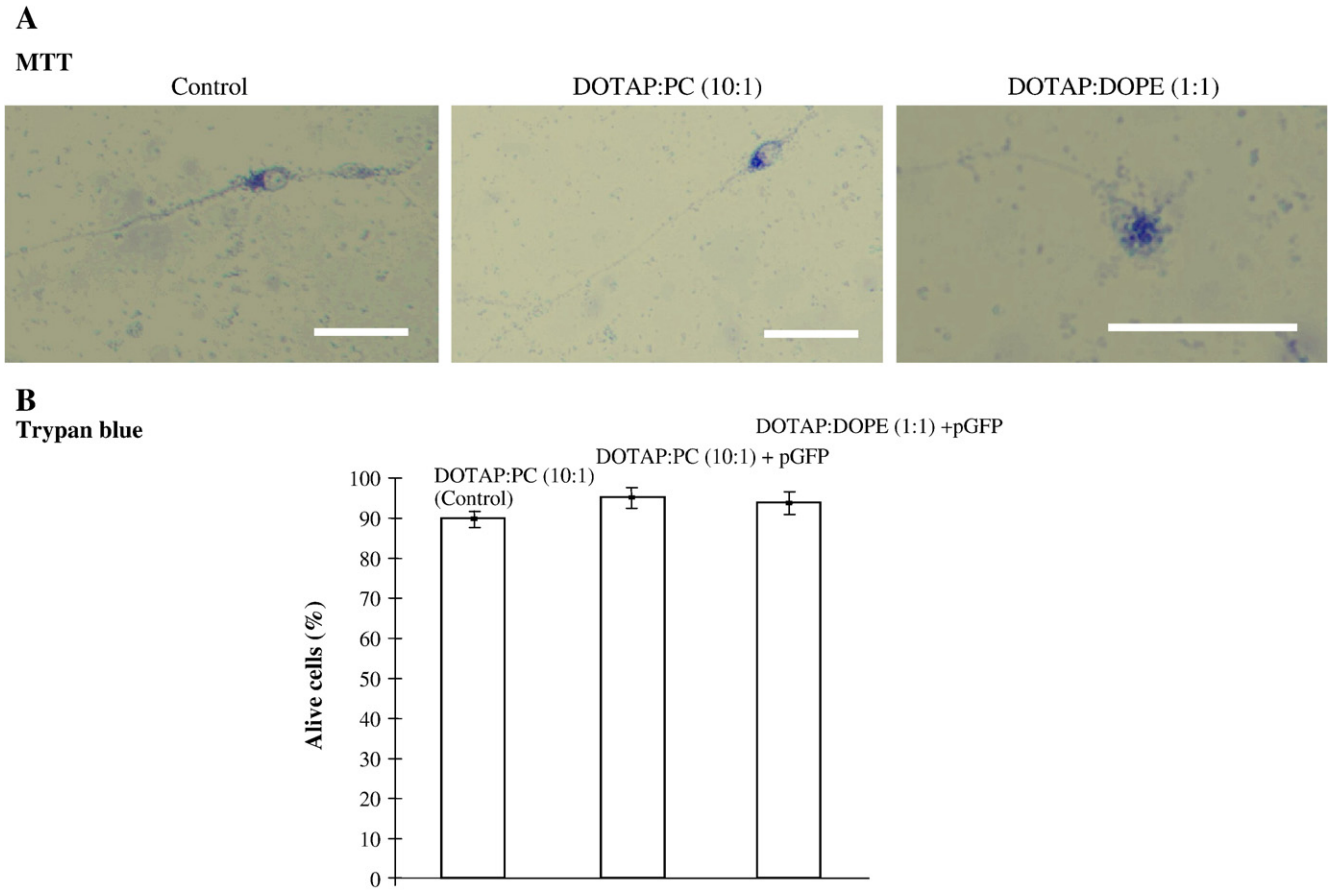

Fig. 3. (A) MTT cell viability assays 48 h after seeding MNs on the coverslips precoated with polylysine/laminin only (control) or plus DOTAP:PC (10:1) or DOTAP:DOPE (1:1). All the cells observed by bright field microscope form purple formazan crystals and thus are alive. Scale bars represent 50 μm. (B) Quantitative analysis of cell viability by Trypan blue exclusion dye assays. Cell counting after 72 h of surfection indicates that neither lipids (Control) nor lipoplex (DOTAP:PC- or DOTAP:DOPE-based lipoplex) deteriorate motoneuron survival. No significant differences were found with ANOVA ($F=1.603$; $df=2$).

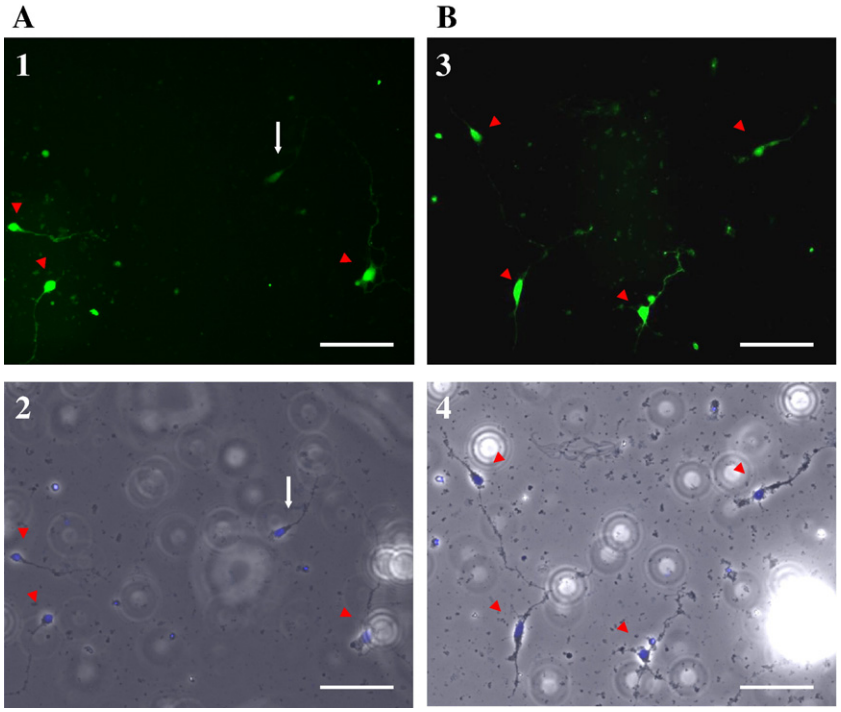

Fig. 4. Fluorescence microscopy of the transient expression of GFP in MNs after 48 h transfection using lipoplex-coated dishes resulting from complexing pGFP with either DOTAP:DOPE (1:1) (A) or DOTAP:PC (10:1) (B): GFP^+ (1, 3) (red arrowhead) and auto-fluorescence (white arrow); Hoechst 33342 fluorescence and phase-contrast micrograph overlays (2, 4). GFP Fluorescence signal has been amplified by antibody directed against to GFP (FITC; green fluorescence). Scale bars represent 75 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorescence assessment. Measurement of GFP fluorescence per dish indicated: 306±6 and 365±8 for low and 3/4 confluence respectively (Fig. 7A). Hoechst 33342 fluorescence was measured and the GFP/Hoechst 33342 fluorescence ratio was calculated for each sample. The ratio was assimilated to "GFP fluorescence per cell". Results in Fig. 7B indicate: 0.87±0.02 and 0.68±0.01 for low and 3/4 confluence, respectively. Control cells were used for evaluating auto-fluorescence. Results in Fig. 7B indicate: 0.12±0.02 and 0.11±0.02 for low and 3/4 confluence, respectively. ANOVA analysis ($F=624.1$; $df=3$) gives a significant result, $^*P<0.001$.

These data clearly show that the lipoplex-coated dish method is better adapted for cell lines seeded at low density than at 3/4 confluence.

DOTAP:PC versus DOTAP:Chol or DOTAP:DOPE

GCL lines were seeded at low confluence and transfection was followed for 72 h. We tested three neutral lipids (PC, DOPE and Chol) associated with DOTAP. We used the same lipid proportion i.e. 1:1 for the three vectors and the fourth we tested DOTAP:PC also at 10:1.

Cell counting indicated: 32.56±3.25%, 38.36±3.54%, 65.07±4.68% and 67.29±2.78% of GFP-positive cells for DOTAP:PC (1:1), DOTAP:Chol (1:1), DOTAP:PC (10:1) and DOTAP:DOPE (1:1) (Fig. 8). ANOVA test ($F=25.78$; $df=3$) reveals significant differences between DOTAP:PC (1:1) versus DOTAP:PC (10:1) ($) and DOTAP:DOPE (1:1) (€); DOTAP:Chol (1:1) versus DOTAP:PC (10:1) (£) and DOTAP:DOPE (1:1) (#) ($^{\$,\ €,\ £,\ \#}P<0.05$).

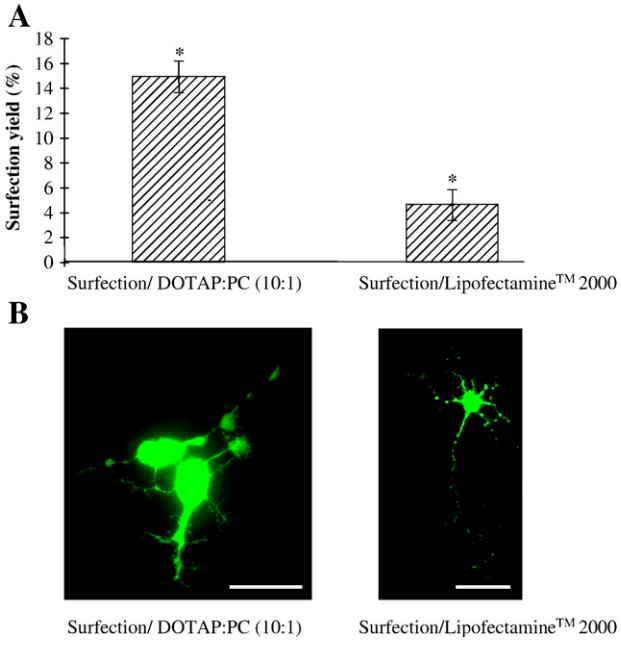

Fig. 5. (A) Surfection yield obtained with DOTAP:PC (10:1) and Lipofectamine™ 2000 and the corresponding fluorescence micrographs. Cell counting after 48 h transfection indicates that DOTAP:PC (10:1) used in surfection method is better than Lipofectamine™ 2000. (B) Data are means±SEM obtained from nine dishes of three independent experiments. Student's *t*-test analysis gives $t=5.965$ (Scale bars represent: 20 μm).

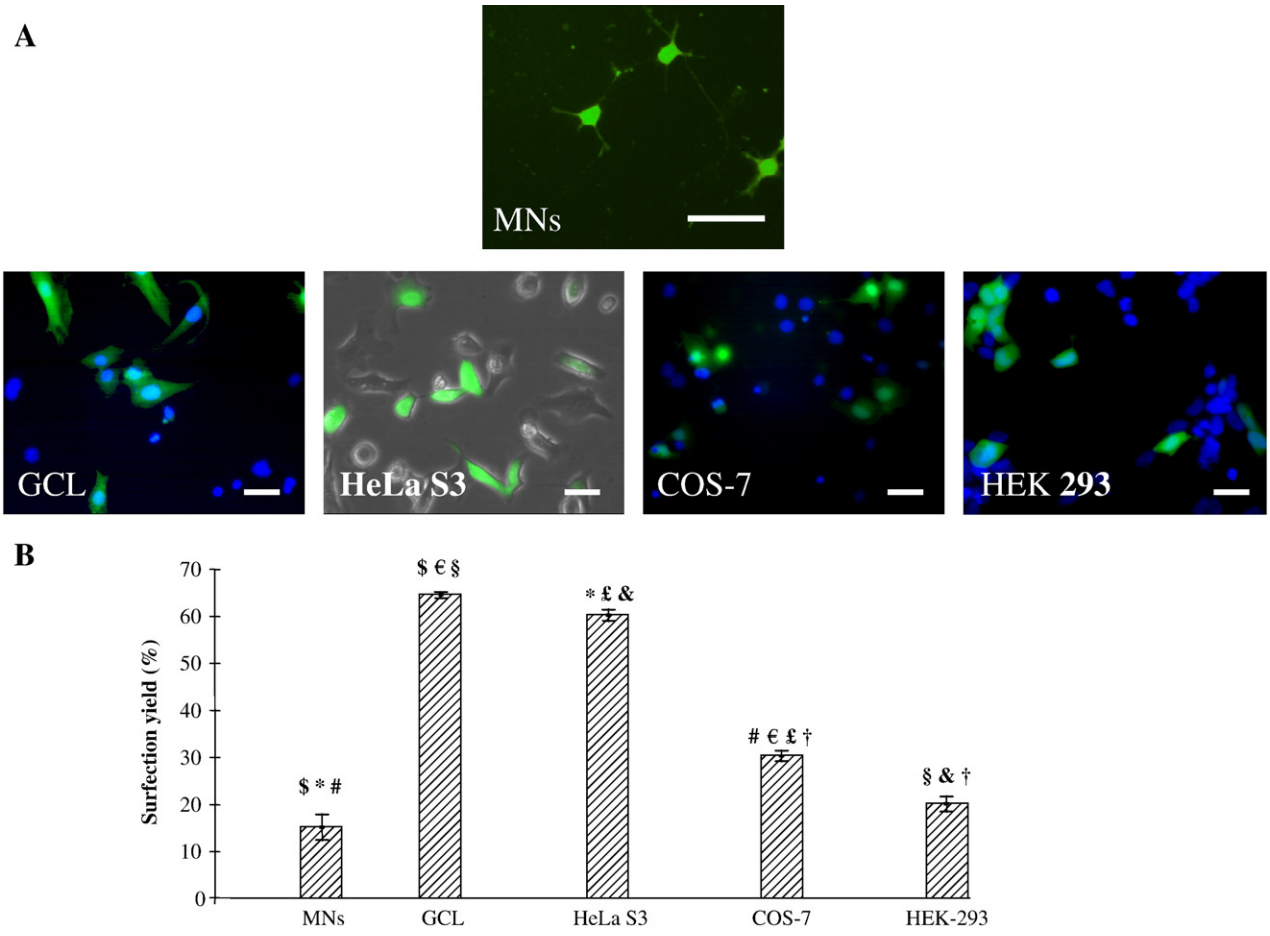

Fig. 6. (A) Surfection yield obtained with DOTAP:PC (10:1) lipids complexed with pGFP. Cells were transfected for 72 h at low density. We observed more GFP^+ cells (green) in GCL and HeLa S3 cultures than in COS-7 and HEK 293 cultures. (B) Data resulting from cell counting are means ± SEM obtained from nine dishes of three independent experiments. ANOVA analysis ($F=187.5$; $df=4$) reveals significant differences between: MNs versus GCL, HeLa S3 and COS-7 ($, *, #), GCL versus HeLa S3, COS-7 and HEK 293 (€, §), HeLa S3 versus COS-7 and HEK 293 (£, and) ($^{\$, *, \#, €, §, £, \&}P>0.02$) and COS-7 versus HEK 293 ($^{\dagger}P<0.02$). Scale bars in A represent 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These data demonstrate that DOTAP:PC (10:1) is as efficient as DOTAP:DOPE (1:1) for the transfection of GCL.

DOTAP:PC 10:1 versus Lipofectamine™ 2000

We compared the two lipid vectors in surfection condition on HeLa cells with Lipofectamine™ 2000 using the classical method as a control. Cells were seeded at mid-confluence. Cell counting of fluorescent GFP-positive cells after 72 h indicates: 72.00 ± 0.75% of transfected cells for Lipofectamine™ 2000 surfection, 62.04 ± 1.31% for Lipofectamine™ 2000 with the classical method and 60.14 ± 1.00% for DOTAP:PC (10:1) surfection (Fig. 9A). ANOVA analysis ($F=39.46$; $df=2$) reveals significant differences only for surfection by Lipofectamine™ 2000 versus surfection by DOTAP:PC (10:1) and surfection by Lipofectamine™ 2000 versus Lipofectamine™ 2000 in classical method ($^{\$,*}P<0.05$).

Mimicking biological activities by the surfection method

Kennedy's disease or spinal bulbar muscular atrophy is caused by an abnormally long stretch of CAG repeats in the gene coding for the androgen receptor. Expression of this mutated receptor ($_m$AR) in neuronal cell lines has been shown (Lieberman et al.,

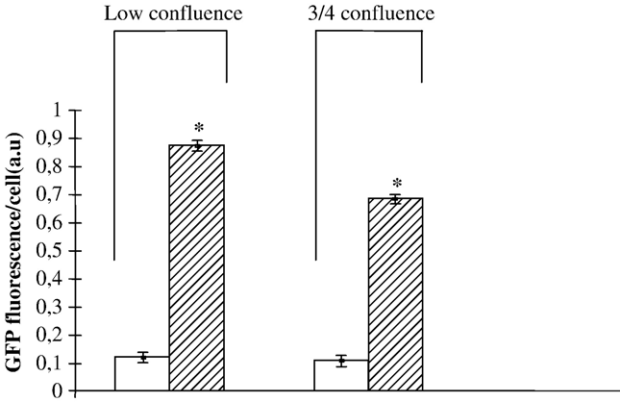

Fig. 7. The influence of the cell density (low and 3/4 confluence) on the efficiency of the lipoplex-coated dish method in the GCL cell lines. Lipoplex was pGFP complexed with DOTAP:PC (10:1). After 24 h of transfection, cells were harvested mechanically with 1 ml of PBS solution and fluorescence measured. The data indicate that low confluence is the best condition for transfecting cell lines with our method. Normalised histograms obtained by plotting the GFP/Hoechst 33342 fluorescence ratio: control cells (white); transfected cells (striped). Data are means ± SEM obtained from nine dishes of three independent experiments and ANOVA analysis ($F=624.1$; $df=3$) gives a significant result, $*P<0.001$.

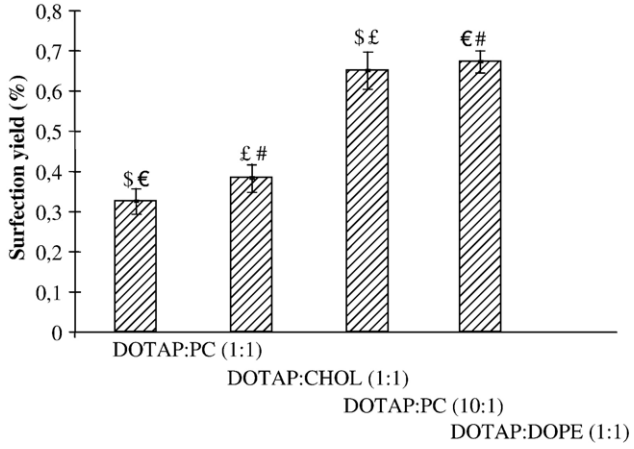

Fig. 8. Surfection yield in the GCL line. GCL lines were seeded at low confluence and transfection stopped after 72 h. Cell counting indicated: 32.56±3.25%, 38.36±3.54%, 65.07±4.68% and 67.29±2.78% of GFP-positive cells for DOTAP:PC (1:1), DOTAP:Chol (1:1), DOTAP:PC (10:1) and DOTAP:DOPE (1:1) (Fig. 10). ANOVA test ($F=25.78$; $df=3$) reveals significant differences between: DOTAP:PC (1:1) versus DOTAP:PC (10:1) ($) and DOTAP:DOPE (1:1) (€); DOTAP:Chol (1:1) versus DOTAP:PC (10:1) (£) and DOTAP:DOPE (1:1) (#) ($^{\$, €, £, \#}P<0.05$). Data demonstrate that DOTAP:PC (10:1) is as efficient as DOTAP:DOPE (1:1) for the transfection of GCL (no significant difference between the two).

2002) to cause formation of polyglutamine-containing aggregates. These authors demonstrate that after transfection with plasmids coding for wild-type and mutated androgen–GFP proteins, in the absence of steroid–hormone in the culture medium, the androgen receptor is found in the cytoplasm. It was further demonstrated that the $_{m}$AR formed cytoplasmic aggregates.

We tested our surfection method on biological and pathological activities of $_{wt}$AR and $_{m}$AR.

Coverslips were coated with lipoplex consisting of a GFP–$_{wt}$AR plasmid and DOTAP:PC (10:1) mixture on which HeLa cells were seeded at low density in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% desteroided fetal calf serum. After 24 h of transfection, we observed a cytoplasmic location of the fluorescent–GFP in living (Fig. 10.A1) and fixed HeLa cells (Fig. 10.A2). Fig. 10. A3 shows nuclear staining with Hoechst 33342.

Then, the same experiment was carried out in HeLa S3 and COS-7 cells with GFP–$_{m}$AR. Cells were fixed 48 h after transfection. We observed cytoplasmic fluorescent-aggregates in HeLa S3 (Fig. 10.B4, B5) and COS-7 (Fig. 10.B6, B7) cells.

Discussion

The aim of this study was to develop a transfection technique best suited for fragile cells such as motoneurons in primary

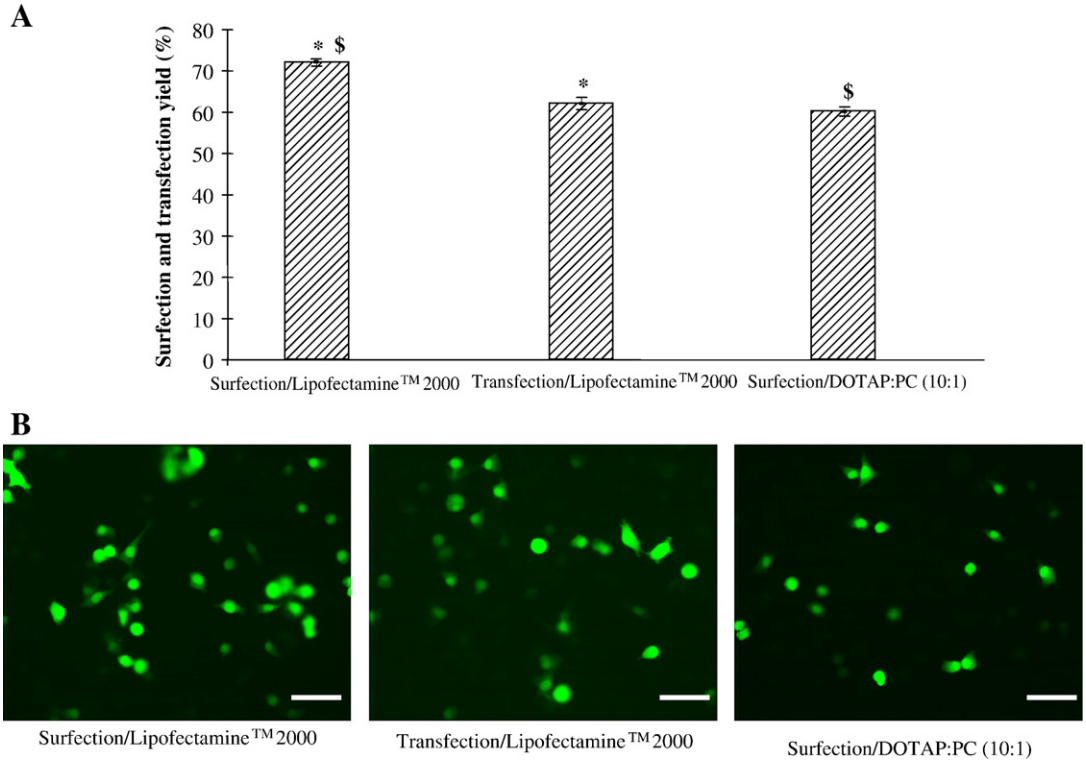

Fig. 9. Comparative analysis of the counting GFP-positive HeLa S3 cells after surfection with Lipofectamine™ 2000 or DOTAP:PC (10:1); Lipofectamine™ 2000 in classical method was used as control. Cells were seeded to reach mid-confluence at the beginning of the transfection step. Cells were counted after 48 h of transfection. Lipofectamine™ 2000 used in surfection method is slightly better than DOTAP:PC (10:1) (B) Corresponding fluorescence micrographs are shown in (C). Scale bars represent 150 μm. (A) Histograms represent total cells (white bars) and GFP-positive cells (grey bars). (B) Surfection and transfection yields obtained by plotting the GFP/Total cells per field ratio ×100 (striped bars). Mean±SEM obtained from nine dishes of three independent experiments and ANOVA analysis ($F=39.46$; $df=2$) reveals significant differences only for surfection by Lipofectamine™ 2000 versus surfection by DOTAP:PC (10:1) and surfection by Lipofectamine™ 2000 versus Lipofectamine™ 2000 in classical method ($^{\$,*}P<0.05$).

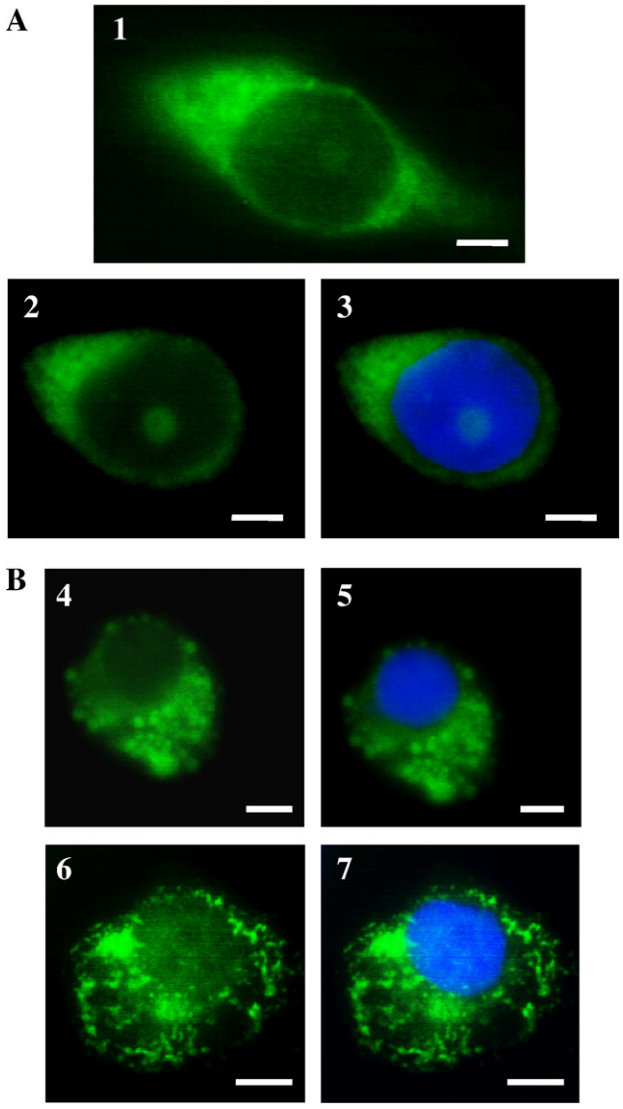

Fig. 10. (A) Specific cytoplasmic localisation of the expressed chimera androgen receptor $GFP_{wt}AR$ when the cells were grown in hormone-deprived culture medium. Initially, before cell seeding the coverslip was coated with a lipoplex composed of pGFP–$_{wt}$AR and DOTAP:PC (10:1). $_{Wt}$AR is the wild-type receptor with 21 CAG at N-terminus. Then, HeLa cells were transfected in DMEM and 10% desteroided serum for 24 h. Fluorescence was observed either directly on living cells (1) or on cells fixed with paraformaldehyde (2). Labelling with Hoechst 33342 stain the nuclei (3). (B) Formation of green fluorescent aggregates in the cells transfected with the mutated androgen receptor. The chimera gene is pGFP–$_{m}$AR. $_{m}$AR is the mutated receptor with 51 CAG. GFP^{+} HeLa S3 (4, 5) and COS-7 (6, 7) cells contained green fluorescent aggregates in their cytoplasm (4, 6). Nuclei were labelled with Hoechst 33342 (5, 7). Scale bars represent 4 μm.

culture, inducing minimal damage. Liposomes are well known as the least toxic transfection agent *in vitro* or *in vivo*. Data have demonstrated the efficiency of DOTAP-based liposome in cell culture and in animal models (Li and Huang, 1997; Crook et al., 1998). In our preliminary studies we tested the DOTAP-based lipoplex and another transfection agent, Lipofectamine™ 2000, in suspension according to current methods for transfecting freshly seeded motoneurons and MNs that were in culture for 5 days (data not shown): most of the cell population was detached from the substratum and died after 24 h even if the cells were incubated with the transfection agents for only a short period (2 h).

The substrate-mediated gene transfer methods deliver DNA in a slow-release manner (Shea et al., 1999; Bielinska et al., 2000; Zheng et al., 2000). This is reason why we used this technique for transfecting motoneurons, which are highly sensitive to the minute environmental changes.

Minimal steps were obtained by using polylysine/laminin as adherent molecules and trappers of lipoplex molecules. PLGA or collagen used by Bielinska et al. (2000) are not proper for culture substrata for primary cultures of neuronal cells, in particular motoneurons. Moreover, after time-consuming calibration experiments, this approach needs an additional step: once hardened, the biopolymer must be cut to the size of the coverslip. To avoid this laborious stage, Chang et al. (2004) used a particular mould (Grace Bio-Labs). These methods need to be carried out in sterile conditions, thus adding an additional stage. Furthermore, cultured motoneurons have such a limited survival time that some authors had to increase their survival by transfecting them with gene coding for growth factors (Cisterni et al., 2000), thus demonstrating the difficulties for handling this cell type in culture and pleading for uncomplicated transfection methods.

The relatively high level of auto-fluorescence (mean: 17%) made it difficult to estimate the number of GFP-positive MNs reliably. Therefore we used for quantification: (i) either a fluorometer or (ii) anti-GFP antibody labelling for counting transfected motoneurons (Fig. 5).

Our choice of the auxiliary lipids to test in association with DOTAP was made on simple grounds. Because about 80% of the plasma membrane is made of PC, this lipid is an obvious choice. Next we have tested two other lipids, DOPE and cholesterol that have been used by Da Cruz et al. (2004), to transfect cell lines and neurons in primary cultures. A second reason to test DOPE lies in its fusogenic properties. It has been demonstrated that DOTAP:DOPE adopts a structure called reversed hexagonal phase (Farhood et al., 1995). Because of this structure, the lipoplex is not retained long inside the endosome after endocytosis. Therefore, it is reasonable to suppose that less lipoplex is degraded.

Combined data (cell counting and quantitative analysis) (data not shown) obtained with both DOTAP:PC (10:1) and DOTAP:DOPE (1/1) vectors indicated a transfection efficiency of at least 15% for MNs in primary culture with significant improvement for the DOTAP:PC (10:1) system. However, cholesterol as an auxiliary lipid appeared incompatible with our lipoplex coating method. It is known that the cholesterol content determines the fluidity of the membrane. We suppose that the locally elevated presence of cholesterol perturbs the membrane lipid environment of the MNs and thus cell adhesion.

To our knowledge, we are the first to transfect MNs in primary monoculture in a natural way. Haastert et al. (2005) succeeded in transfecting MNs in primary co-culture with Schwann cells using a non-liposomal lipidic formulation (effectene, micelles) in combination with a special DNA-condensing enhancer (neupherin–neuron) with a 1% yield.

The MTT and Trypan blue survival tests confirm that the motoneurons seeded on the culture substratum precoated by the lipoplex are not damaged.

Once we assessed this transfection method for neurons and motoneurons, we checked whether it could be extended with equal success to cell lines, thus allowing standardization of the transfection protocol. We confirmed the performance of our method on several lines: HeLa S3, HEK 293, COS-7 and GCL lines. Also, our experiments with HeLa S3 cells clearly show that whatever the transfection method used, the resulting biological phenomena (localisation of $_{wt}$AR and aggregates containing $_{m}$AR) are the same.

We used polylysine-coated coverslips as a substratum for our cultures. In order to overcome the electric repulsion between polylysine and the lipoplex that are both cationic, we added a negatively charged layer of laminin.

Even-Chen and Barenholz (2000) have shown that DOTAP:DOPE (1:1) bind DNA more strongly than DOTAP:Chol (1:1). Therefore the former is more compact than the latter. Because small particles are more easily taken up by endocytosis than large particles, a vector containing DOPE should enter the cell more easily than a vector containing cholesterol. This could explain the superior transfection efficiency of DOTAP:DOPE (1:1) in our cell lines.

Amongst the obstacles to transfection, serum has been reported to exhibit an inhibitory effect. This is probably due to the presence of negatively charged lipoproteins that interfere with the association of the lipoplex with the cell membrane (Gao and Huang, 1995). The presence of polylysine can diminish this unwanted interaction by competition. Polylysine also improves the capacity of the transfection agent to deliver its transgene because, in its absence, the release of DNA from the lipoplex is hampered by serum protein (Yang and Huang, 1997). Thus, we also used polylysine and laminin to transfect cell lines. We found the best result with the lipid/DNA charge ratio *R* value of 4. Yang and Huang (1997) found the same result when transfection is carried out in the presence of 20% serum, suggesting that the free excess positive charges in the lipoplex neutralize negative charges in the serum protein.

As shown by our data on GCL lines, the transfection rate decreased when cell density increased. One explanation may be the limited number of lipoplex molecules trapped inside the polylysine/laminin network and the fact that they were immobilised in this support, whereas in current methods, the lipoplex molecules can freely circulate for 2–6 h. For this reason, the commercially available lipid vectors are recommended for 3/4 confluence.

We also propose this approach as a ready-to-use technique. We have dried lipoplex-coated coverslips containing pGFP, stored them at +4°C for 6 months and used them successfully, simply after rehydration, to transfect COS-7 cells (data not shown).

In summary, we have developed a new and non-conventional method for transfecting neural cells in primary culture, in particular MNs, using lipoplex-coated dishes. Efficiency of the method termed “mild surfection” has also been demonstrated for cell lines (HeLa S3, HEK 293, COS-7 cells and GCL lines). Our method spares cells during the relatively aggressive transfection step as far as possible, while offering a good yield. We conclude that the transfected cultured MNs provide an original “in vitro” model for studying motoneuronal degenerative diseases, particularly those linked to hormonal receptors.

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