

Efficient transfection of DNA or shRNA vectors into neurons using magnetofection

Thomas Buerli^{1,2,5}, Christophe Pellegrino^{3,5}, Kristin Baer⁴, Barbara Lardi-Studler², Ilona Chudotvorova^{3,6}, Jean-Marc Fritschy², Igor Medina³ & Christian Fuhrer^{1,6}

¹Department of Neurochemistry, Brain Research Institute, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. ²Institute of Pharmacology and Toxicology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. ³INMED/INSERM Unite 29, 163 Route de Luminy, 13009 Marseille, France. ⁴School of Medicine, Institute of Life Science, Swansea University, Singleton Park, Swansea SA2 8PP, UK. ⁵These authors contributed equally to this work. ⁶Present address: Department of Psychiatry, Mount Sinai Medical Center, One Gustave Levy Place, New York, New York 10029, USA (I.C.); Open Access Coordination, Research Library Irchel, Main Library, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland (C.F.). Correspondence should be addressed to I.M. (medina@inmed.univ-mrs.fr).

Published online 29 November 2007; doi:10.1038/nprot.2007.445

Efficient and long-lasting transfection of primary neurons is an essential tool for addressing many questions in current neuroscience using functional gene analysis. Neurons are sensitive to cytotoxicity and difficult to transfect with most methods. We provide a protocol for transfection of cDNA and RNA interference (short hairpin RNA (shRNA)) vectors, using magnetofection, into rat hippocampal neurons (embryonic day 18/19) cultured for several hours to 21 d *in vitro*. This protocol even allows double-transfection of DNA into a small subpopulation of hippocampal neurons (GABAergic interneurons), as well as achieving long-lasting expression of DNA and shRNA constructs without interfering with neuronal differentiation. This protocol, which uses inexpensive equipment and reagents, takes 1 h; utilizes mixed hippocampal cultures, a transfection reagent, CombiMag, and a magnetic plate; shows low toxicity and is suited for single-cell analysis. Modifications done by our three laboratories are detailed.

INTRODUCTION

Every gene transfer method aims to achieve high transfection efficiency, low toxicity and long-lasting expression. These goals are especially difficult to achieve for postmitotic primary neurons due to their high sensitivity to any microenvironmental change^{1–5}. Many currently available transfection methods do not yield sufficiently good gene expression results to allow functional gene analysis in differentiated adherent neurons *in vitro*. A recent report provides a useful overview of seven different gene delivery methods, including two often-used transfection techniques (calcium-phosphate and lipofection), but excluding magnetofection⁶.

Magnetofection is a technique that can be used to reliably and efficiently introduce DNA into a variety of cell types⁷. Therefore, we have invested in designing a reliable, magnetofection-based protocol to express DNA or shRNA constructs in rat hippocampal neurons (embryonic day (E)18/19) cultured for several hours to 21 d *in vitro* (DIV). After transfection, neurons expressing exogenous proteins can develop normally in culture for 5–10 d. We have determined the optimum parameters for transfection efficiency such as age of cells at transfection time point, expression levels during the time after transfection and parameters for double-transfection. This protocol shows low toxicity, uses a combination of a transfection reagent, CombiMag, and magnetic force and shows a sufficiently high efficiency in neurons (ca. 5%) to allow single-cell analysis.

We present a core protocol and illustrate the critical steps, modifications and applications carried out by our three laboratories (Medina, Fuhrer and Fritschy)^{8–10}. This protocol allows single and double transfections of DNA vectors at different time points after plating. First, we present a detailed characterization of magnetofection parameters such as age of neurons, time course of protein expression after magnetofection, and transfection efficiency in pyramidal neurons and GABAergic interneurons. Three specific

applications are then demonstrated. In the first application, expression of the synaptic scaffold protein gephyrin¹¹ was compared after early and late transfection. The slow development of GABAergic synapses proceeded normally under these circumstances, showing that gephyrin-GFP expression did not interfere with neural development and synapse formation. In the second application, we concentrated on two genes, $\alpha 7$ nicotinic acetylcholine receptor and the scaffold protein PICK1¹⁰. We achieved simultaneous expression of these two genes in GABAergic interneurons, which form a small subset of cells. This is quite an achievement, considering how hard neurons are to transfect with most methods. In the third application, long-lasting expression of shRNAs against the synaptic GTPase activating protein (SynGAP)^{12,13} was optimized. We achieved long-lasting decrease of the expression of SynGAP in neurons for up to 6 d. These three applications were successful by bringing about small variations in the cell culture and magnetofection protocol, which are mainly determined by the time point of transfection relative to plating (see Methods A–C in Table 1).

In comparison with the seven recently reviewed methods of gene delivery⁶, we note the following advantages of our magnetofection protocol. First, the method is cost-effective, requires no expensive equipment, is easy to perform, takes only 1 h and does not depend on preparation of complex constructs such as viruses. Second, it is not toxic for neurons, is used on young as well as mature neurons and allows long-term gene expression. Other methods are preferentially used for young neurons (e.g., 4 DIV¹⁴), which are generally much easier to transfect than mature neurons, or, in the case of electroporation and nucleofection⁶, only for cells in suspension at the time of plating. Third, we achieve expression of two mammalian proteins, not only GFP (which is comparatively easy to express), in the same neuron. Most of the other protocols concentrate on only one gene, or one mammalian gene in combination

TABLE 1 | Overview of cell culture conditions, media and concentrations of reagents used, depending on preferred magnetofection time point and as done by the three laboratories (A, Fuhrer and Fritschy laboratories; B, Fritschy and Medina laboratories; C, Medina laboratory).

	Method	A	B	C
Cell culture before magnetofection	Culture dish	18 mm coverslip in a 12-well plate	35 mm dish (containing 3 × 14 mm coverslip)	
	Transfection time point	< 13 DIV	< 10 DIV	> 14 DIV; also possible on younger neurons
	Protein expression	Short-term	Short-term; long-term expression is possible in young neurons	Short-term or long-term
	Number of cells	50,000	400,000–800,000	800,000–1,200,000
	Volume of plating media (adhering step)	200 µl per coverslip	—	—
	Culture media	MEM supplemented with 15% Nu-serum, 2% B27, 15 mM HEPES (pH 7.2), 0.45% glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 IU ml ^{−1} penicillin–streptomycin	MEM supplemented with 10% Nu-serum, 15 mM HEPES (pH 7.2), 0.45% glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 IU ml ^{−1} penicillin–streptomycin	Until 4 DIV, MEM supplemented with 10% Nu-serum, 15 mM HEPES (pH 7.2), 0.45% glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 IU ml ^{−1} penicillin–streptomycin was used
	Media volume	2 ml per well in a 12-well plate		2 ml per 35 mm dish
	Feeding media	Culture medium remains, no media exchange	Culture medium remains, no media exchange	From 5 DIV, gradual exchange of the culture medium with a feeding medium (MEM supplemented with 2% B27, 15 mM HEPES, 0.45% glucose, 1 mM sodium pyruvate and 2 mM L-glutamine). Exchange 50% of medium with fresh feeding medium; repeat every third day and the day before transfection
Magnetofection	DNA (A)	1 µg in 30 µl OptiMEM		0.8 µg in 150 µl OptiMEM
	Lipofectamine 2000 (B)	2 µl in 30 µl OptiMEM		7 µl in 150 µl OptiMEM
		Incubate for 5 min at room temperature; combine (A) and (B)		
	CombiMag	2 µl, 1:10 dilution in H ₂ O		1 µl, undiluted
		Add CombiMag, mix and incubate for 15 min at room temperature		
	Cells	Remove 50% medium and save it; leave 1 ml in each well		Leave media
Assay		Carefully add transfection complex to cells		
		Incubate cells on magnetic plate at 37 °C, 5% CO ₂ for 25 min		
	Media change	Replace by saved medium		Replace 80% by feeding media
		Perform assay earliest after 6 h		

with GFP¹. Fourth, the overall efficiency of our method is comparable to the recently reviewed calcium-phosphate and lipofection methods⁶. However, we achieve expression of two mammalian

genes in a small subpopulation of cells, the GABAergic interneurons. This shows that even small neuronal cell subpools are unlikely to be excluded from gene expression in our approach.

BOX 1 | DNA CONSTRUCTS

The DNA constructs used in our applications were the following:

- (i) pEGFP (Clontech 6077-1)
- (ii) Full-length gephyrin derived from pcDNA3-based constructs²³, PCR-cloned into the *EcoRI/SalI* site of pEGFP-C2 (Clontech; a gift from Dr. Günter Schwarz, University of Cologne)
- (iii) C-terminally Flag-tagged mouse $\alpha 7$ nAChR in pCS2⁺ expression vector (a gift from Dr. Ines Ibanez-Tallon, MDC Berlin-Buch)
- (iv) N-terminally EYFP-tagged rat PICK1 in pRK5 expression vector (PICK1-EYFP) and the empty control EYFP vector (a gift from Prof. Ann Marie Craig and Dr. Fernanda Laezza, Washington University²⁴)
- (v) SynGAP shRNA created by insertion of the following oligonucleotides into the *Hpa1/Xho1* sites of the Lentilox 3.7 vector (ATCC; no VRMC-39)^{21,22}:
 5'-TGAAGCGATATTACTGCGAGTTCAAGAGAACTCGCAGTAATATCGTTCTTTTTC-3'
 5'-TCGAGAAAAAGAAGCGATATTACTGCGAGTTCTTGAACCTCGCAGTAATATCGTTCA-3'

MATERIALS

REAGENTS

- One timed-mated rat (Wistar) **! CAUTION** All experiments using live rodents should conform to local and national regulations.
- PBS (Gibco, 18912-014)
- Minimum essential medium (MEM; Invitrogen, 31095-052)
- Dulbecco's modified Eagle's medium (DMEM; Gibco, 61965-026)
- Nu-serum (Becton Dickinson, 355500)
- B27 supplement (Invitrogen, 17504044)
- FCS (Gibco, 10270-106)
- BSA (Sigma, A-9418)
- Papain (Sigma, P-4762)
- DNase I (Sigma, DN25)
- Glucose monohydrate (Mw 198.7 Da)
- 1 mM sodium pyruvate (Invitrogen, 11360-039)
- 1 M HEPES (Invitrogen, 15630056)
- 2 mM L-glutamine (Gibco, 25030-24)
- 10 IU ml⁻¹ penicillin-streptomycin (Gibco, 15140-122)
- OptiMEM (Invitrogen, 31985-070)
- CombiMag 100 (OZ Biosciences, CM20100)
- Lipofectamine 2000 (Invitrogen, 11668-019)
- Poly-L-lysine (Sigma, P-1520)
- DNA constructs (generated according to standard molecular biology techniques, those we used are in **Box 1**)
- Endo-free Maxi prep purification kit (Qiagen, 12262)
- 0.25% trypsin (wt/vol; Gibco, 25090-028)
- 45% D-glucose (wt/vol; Sigma, G-8769)

EQUIPMENT

- Standard cell culture equipment (laminar flow hood, dissecting tools, incubators, hoods, centrifuges, water baths, fridge and freezers)

- Molecular biology equipment (fridge, freezers, oven, sonicator, centrifuges, pipettes, plastic ware, tips and glassware)
- Magnetofection apparatus including super magnetic plate (OZ Biosciences, 10000)
- 18 mm glass coverslips coated with poly-L-lysine (Menzel)
- 14 mm microscope coverslips (Marienfeld GmbH & Co, VWR 0111520)
- 12-well tissue culture plates (NUNC or Greiner)
- 15 ml Falcon tubes
- Pasteur pipettes
- Neubauer counting chamber of 0.1 mm depth, 0.0025 mm² (Optik Labor)
- Inverted fluorescence microscope equipped with long-distance air objective ($\times 10$ or $\times 20$ lens)
- Epifluorescence microscope with objective lenses (e.g., Axioskop 2 or ApoTome, Carl Zeiss AG)

REAGENT SETUP

Culture medium For Method A, use MEM supplemented with 15% (vol/vol) Nu-serum, 2% (vol/vol) B27, 0.015 M HEPES (pH 7.2), 0.45% (wt/vol) D-glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 IU ml⁻¹ penicillin-streptomycin. For Methods B and C, use MEM supplemented with 10% (vol/vol) Nu-serum, 15 mM HEPES (pH 7.2), 0.45% (wt/vol) D-glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 IU ml⁻¹ penicillin-streptomycin. See **Table 1**.

Feeding medium For Method C, use MEM supplemented with 15 mM HEPES, 0.45% (wt/vol) D-glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 2% (vol/vol) B27. See **Table 1**.

Borate buffer 0.15 M boric acid, pH 8.4.

Papain solution PBS with 10 mM glucose containing 0.05% (wt/vol) papain and 0.1% (wt/vol) BSA.

Dissection solution PBS with 0.25% (wt/vol) D-glucose.

PROCEDURE

Preparation of glass coverslips

1| Dissolve poly-L-lysine stock in water to obtain a 1 mg ml⁻¹ solution, distribute in 1 ml aliquots and store at -20°C .

2| Prepare cover glasses as follows. Place coverslips into a 200 ml flask with narrow neck. Shake them overnight in 1% (vol/vol) nitric acid. Rinse the coverslips thoroughly (shake for at least 3 h in tap water; change water every 20–30 min). Sonicate coverslips three times for 30 min (using, e.g., Fisher ultrasonic cleaner 15-335-140) in distilled water, then three times in 70% (vol/vol) ethyl alcohol and, finally, two times in 95% (vol/vol) ethyl alcohol. Place the washed coverslips in a glass Petri dish and sterilize at 160°C for 1 h.

■ PAUSE POINT Pre-washed sterile coverslips can be stored at room temperature ($\sim 20^{\circ}\text{C}$) for a few months.

3| Coat cover glasses with poly-L-lysine: Dissolve the stock of poly-L-lysine in distilled water or borate buffer (1:100), filter and apply to culture dishes with coverslips. Incubate overnight at 37°C . Rinse with PBS at least three times.

▲ CRITICAL STEP Such rigorous procedures of washing and coating of coverslips are critical for neuron culturing. Insufficient washes of coverslips before coating and/or shortening of the time of coating will result in neuron clustering and suboptimal cultures. Residual poly-L-lysine (insufficient washing) is toxic for neurons.

■ PAUSE POINT Dissolved poly-L-lysine can be stored at 4°C for up to 1 month.

Preparation of solutions

- 4| Dissolve DNase in distilled water to obtain a 10 mg ml⁻¹ solution; filter and store at -20 °C in 0.3 ml aliquots.
- 5| Prepare fresh dissection solution and keep at 4 °C.
- 6| Prepare papain solution and pre-incubate at 37 °C. Before use, add 0.01 mg ml⁻¹ DNase I and sterilize by filtration.
- 7| Prepare culture medium according to **Table 1** (Methods A–C).
▲ CRITICAL STEP Always prepare fresh culture medium.
- 8| Prepare feeding medium if necessary according to **Table 1**.
▲ CRITICAL STEP Feeding medium does not contain any Nu-serum or antibiotics. Feeding medium can be stored at 4 °C for up to 1 week.

Culturing cell type of interest

- 9| Prepare the cell type of interest. This protocol describes the use of rat E18–19 hippocampal neurons. Harvest E18–19 hippocampi and dissect the hippocampi free of meninges in cooled (2–3 °C) dissection solution.
▲ CRITICAL STEP It is essential to remove all the meninges from the hippocampi.
▲ CRITICAL STEP The temperature of the dissection solution is critical; it should not be more than 2–3 °C.
▲ CRITICAL STEP Optimization of neuronal cultures is an essential requirement for the success of this method. The key point to be noted is that glia–neuron coculture using defined medium has to be used, and not Banker type cultures nor glia feeder layer (in Banker cultures, neurons are separated from a glia feeder layer but are immersed in the same medium, which has a different composition than ours^{15,16}). All our attempts to modify this protocol gave negative results. Also, we failed to transfect neurons prepared using other protocols, particularly those grown in neurobasal media.
▲ CRITICAL STEP In our experience, the batch of Nu-serum varied, so it is essential to do a titration experiment with different Nu-serum concentrations (e.g., 10%, 15%, 20% (all vol/vol)).
- 10| Incubate hippocampi with papain solution for 15 min at 37 °C; gently shake every 5 min. For example, we routinely use 10–20 hippocampi in 5 ml of solution in one 15 ml Falcon tube.
▲ CRITICAL STEP You can replace papain with trypsin (final concentration 0.25%), as both give similar results. The critical point is the time of incubation.
- 11| Stop the papain reaction by washing twice with DMEM and then resuspend in a final volume of 1 ml of DMEM/FCS (10% vol/vol).
- 12| Triturate the tissue using a fire-polished Pasteur pipette ten times. Transfer the dissociated cells into a new tube.
! CAUTION We recommend gentle trituration of the tissue for exactly 10 times. The diameter of the fire-polished Pasteur pipette should be adjusted to result in single-cell dissociation without rupturing the cells by turbulences.
- 13| Calculate the number of neurons using a Neubauer plate. Place a 10 µl sample of the cell suspension onto the Neubauer plate and cover it with a cover glass. Capillary action completely fills the chamber with the sample. Count the cells in an area of 1 mm² (equals a volume of 100 nl) using a microscope. Multiplying the number of counted cells by 10⁴ gives the number of cells per milliliter.
- 14| Dilute the dissociated neurons to the desired concentration with culture medium. Plate cells at a density according to **Table 1**. In Method A, the plating volume is 200 µl per coverslip with 50,000 cells. In Methods B and C, dilute the cell suspension to a desired concentration (200,000–600,000 cells per ml) and directly apply 2 ml of suspension to the 35 mm dish.
! CAUTION We recommend optimization of the number of cells relative to the dish/coverslip used. Usually from ten embryos, we prepare 20–25 culture dishes (35 mm).
- 15| Let the cells adhere to the coverslip for 1 h at 5% CO₂/37 °C. If using 18 mm coverslips in 12-well plates (Method A, **Table 1**), transfer the coverslip to the 12-well tissue culture plate and add 2 ml of culture medium per well; in our experience, more medium is toxic to cells. This step is not required for 35 mm dishes.
- 16| Grow the cells at 5% CO₂ and 37 °C, until the desired time point for transfection.
▲ CRITICAL STEP Optimal neuronal growth before magnetofection is a key parameter for successful experiments. It depends on the initial cell density, pH of the culture medium (7.2; verify that the incubator has the correct %CO₂ settings) and medium supplementation with Nu-serum and B27. In particular, problems with astrocyte overgrowth can be solved by varying the concentration of Nu-serum.
▲ CRITICAL STEP In good-quality cultures, neurons are equally distributed throughout the dish or coverslip during the whole period of culturing. Aggregation of neurons in clusters after 7–10 DIV markedly affects transfection efficacy. Also, neurons growing in clusters might have different properties (synapse density) as compared to neurons growing as a monolayer.

▲ CRITICAL STEP For short-term transfection of mature neurons (10–12 DIV; Method A in **Table 1**), the initial cell density is relatively low and addition of B27 to the culture medium is required to stimulate neuronal growth. We recommend leaving the cells in the same culture medium for the entire time from before magnetofection up to 12 DIV. In our experience, the cells suffer from repeated medium changes.

▲ CRITICAL STEP Specific comments for Methods B and C: for magnetofection of immature neurons (6 DIV; Method B in **Table 1**), it is better to start with a density of 600,000 cells per 35 mm dish and without B27; if transfection is long-term (> 4 d), a feeding medium containing B27 is required after transfection (Method C in **Table 1**); finally, for late magnetofection (> 14 DIV; Method C in **Table 1**), a higher initial cell density is necessary (800,000 cells per 35 mm dish), and the culture medium is replaced gradually with a feeding medium containing B27. Start feeding the neurons at 5 DIV and change 50% of the medium every 3 d. Twenty-four hours before transfection, replace 50% of the culture medium with fresh medium.

▲ CRITICAL STEP Best results with Methods B and C are obtained by growing cells on 14 mm coverslips placed in 35 mm plastic dishes. Place these culture dishes into a larger (150 mm) Petri dish to prevent evaporation of the culture medium. Incubate cultures in the CO₂ (5%) incubator at 37 °C.

? TROUBLESHOOTING

Magnetofection procedure

17| Prepare or obtain DNA constructs of interest.

▲ CRITICAL STEP The use of high-quality DNA obtained with Maxi prep kits and additional purification with phenol/chloroform improves transfection efficacy.

18| Prepare transfection mixture. The following procedure applies for both cDNA and shRNA vectors. First combine DNA and OptiMEM in one Eppendorf tube (solution A). Different concentrations of reagents are required for Method A and for Methods B and C (see **Table 1**).

▲ CRITICAL STEP Double-magnetofection can be difficult; do not exceed a total of 1 µg DNA, as it considerably decreases the rate of transfection.

19| Combine lipofectamine 2000 and OptiMEM according to **Table 1** in a second Eppendorf tube (solution B), mix gently and incubate solutions A and B for 5 min at room temperature (20 °C).

20| Combine solutions A and B.

21| For 18 mm coverslips, dilute CombiMag in H₂O (1:10) and vortex the diluted solution; for 35 mm dishes, use undiluted CombiMag solution and vortex (see **Table 1**).

▲ CRITICAL STEP You can keep the diluted CombiMag solution for up to 1 month at 4 °C, but we do not recommend use of older solutions.

22| Add CombiMag solution to the combined solutions A and B (CombiMag–DNA–lipofectamine 2000), mix and incubate for 15 min at room temperature (see **Table 1**).

▲ CRITICAL STEP Gently handle the DNA transfection complex.

23| For 18 mm coverslips (Method A, **Table 1**), take the 12-well dish containing cultured neurons out of the incubator and remove half of the culture medium. Leave 1 ml per well. Store the removed medium.

▲ CRITICAL STEP Ensure that the cultured neurons are on the top of the coverslip.

24| Carefully add CombiMag–DNA–lipofectamine 2000 complex dropwise to cells.

25| Incubate cells on a pre-warmed magnetic plate at 37 °C for a minimum of 25 min.

▲ CRITICAL STEP The transfection time on the magnetic plate is a critical point. In our experience, longer transfection time results in higher transfection efficiency, but often higher toxicity.

▲ CRITICAL STEP Transfection efficiency is dependent on the plasmid backbone.

26| Replace the medium including CombiMag–DNA–lipofectamine 2000 complex (see **Table 1**).

▲ CRITICAL STEP In immature neuronal cultures (< 10 d), the entire medium can be replaced with saved pre-warmed culture medium or feeding medium (never let neurons dry). In older neurons, complete removal of the medium will damage neurons. To avoid this, we change 80% of the medium.

▲ CRITICAL STEP For some constructs, protein expression could be optimized by decreasing the incubator temperature to 32 °C.

? TROUBLESHOOTING

27| Transfer the cells to the incubator for at least 6 h.

? TROUBLESHOOTING

Assay and analysis

28| Perform assay and analysis (immunocytochemistry, electrophysiology or image analysis) according to your experimental setup, at the earliest 6 h after transfection. We routinely perform immunocytochemistry and capture images using epifluorescence microscopy (Axioskop 2 or ApoTome, Carl Zeiss AG) combined with a high-resolution digital camera (Hamamatsu Photonics) or by confocal laser-scanning microscopy (TCS 4D; Leica; Fluoview 500, Olympus). Collect high-resolution confocal laser-scanning microscope images ($\times 63$ or $\times 100$ oil immersion objective) of neurons, fluorescently double-labeled for the proteins of interest. Contrast-optimize these images using imaging software (e.g., using Adobe Photoshop, Adobe Systems Inc.). Perform quantitative analysis using imaging software, keeping constant threshold levels (e.g., using ImageJ, NIH; or Metamorph).

● TIMING

Steps 1–3, preparation of glass coverslips: 1 d
Steps 4–8, preparation of solutions: 1–2 h
Steps 9–16, culturing cell type of interest: 8 h–21 d
Steps 17–27, magnetofection procedure: 1 h
Step 28, assay and analysis: 12 h–14 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solutions and suggestions
16	Neuronal cultures show unhealthy morphology	Suboptimal culturing conditions	Essential requirements: optimization of primary neuronal cultures from hippocampus and cortex. Key points: control CO ₂ and pH, control Nu-serum concentration, regularly verify the CO ₂ settings of the incubator
	Too many glial cells/few neurons in the culture	Suboptimal culturing conditions	Vary the amount of Nu-serum (neurons tolerate an increase up to 20% in Method A)
	Neurons in culture form clusters	Appearance of clusters of neurons after 6–10 DIV indicates (i) inefficient coverslip coverage with poly-L-lysine, (ii) insufficient washing of coverslips, (iii) inappropriate glass quality for neuronal cultures	The simplest way to identify the cause of neuronal cluster appearance is to verify the culture quality growing on the plastic between coverslips. If the culture is homogeneous, it is necessary to prepare a new batch of coverslips Coat the coverslips carefully with poly-L-lysine Wash the coverslips properly during preparation (Steps 2 and 3) Use coverslip of some other manufacturer
26	Low magnetofection efficiency of neurons	Suboptimal age of cells at magnetofection time point, suboptimal culturing medium, suboptimal DNA concentration, suboptimal DNA construct, insufficient transfection efficiency	Optimizing the magnetofection parameters is a key element to achieve high and stable magnetofection rates and healthy neurons The age of neurons at the magnetofection time point is a very critical point. In our experience, hippocampal cells cultured according to Method A are well magnetofectable until 11 DIV; afterward, the efficiency drops The culturing medium plays an essential role in achieving high magnetofection rates Use fresh reagents DNA constructs show varying success rates in magnetofection efficiency. Try pEGFP construct from Clontech as a control For every culture type and after changing culturing conditions (new batches of MEM, Nu-serum, lipofectamine 2000, etc.), it is necessary to select the optimal amount of DNA and lipofectamine 2000 used for magnetofection Transfection efficiency depends also on the strength of the magnetic field. Petri dishes are thinner than 12-well plates and might allow higher transfection rates



PROTOCOL

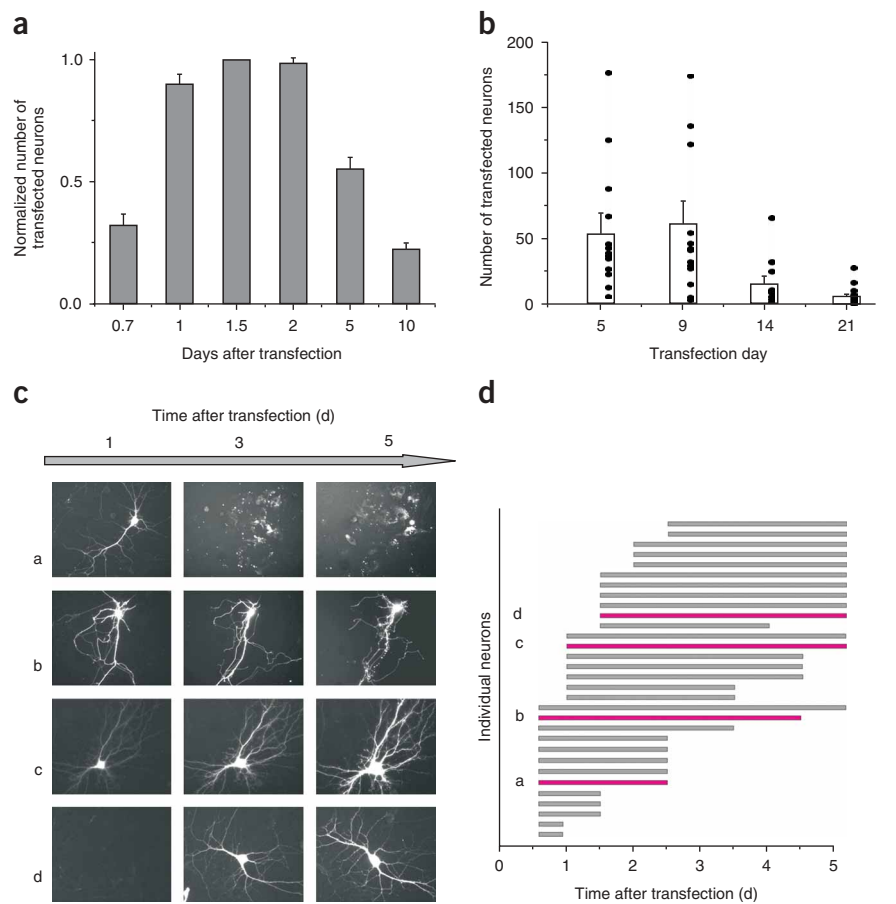
TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solutions and suggestions
	Toxicity of magnetofection on neurons/survival rate of neurons	Suboptimal DNA concentration	We experienced that the DNA concentration as well as the DNA construct itself has an influence on magnetofection efficiency and toxicity
	Low efficiency with double-magnetofection procedure		For routine experiments, do not exceed a total of 1 µg DNA. Do not incubate cells on pre-warmed magnetic plate at 37 °C for longer than 25 min. Magnetofection with 2 µg DNA for longer than 25 min will increase transfection efficiency and also cell toxicity
27	Neurons die after transfection	Usually, neuronal death after transfection is related to medium change and not to toxicity of reagents	We recommend ‘transfecting’ one dish by omitting DNA and lipofectamine from the transfection mixture. If neurons survive the medium change, but die in dishes with applied reagents, decrease the volume of lipofectamine 2000 and/or take another preparation of DNA vector
	After transfection, neurons are alive but there are no transfected cells		Transfect using the same protocol, confluent HEK293 cell cultures with and without incubation step on magnetic plate. The transfection efficacy should be at least 5% after cell incubation without magnetic plate and 20% with magnetic plate. Lower efficacy will suggest the need to change lipofectamine 2000 or DNA

ANTICIPATED RESULTS

We present, as examples, results from three independent groups showing that neuron transfection can be efficiently achieved using magnetofection. We have successfully transfected a variety of DNA and shRNA constructs, most of which were enhanced

Figure 1 | Expression of EGFP in neuronal cultures (Method C). **(a)** Relative number of neurons expressing EGFP at different times after magnetofection at 9 DIV. All transfected neurons were quantified on coverslips at different time points in the same dishes, using a fluorescence microscope equipped with CO₂, camera and temperature controller. Between acquisitions, cultures were kept in the CO₂ incubator. Data from seven experiments are shown. **(b)** Number of transfected neurons per 14 mm coverslip as function of culture age. EGFP fluorescent neurons were quantified 36 h after magnetofection. **(c)** Images of the same fluorescent neurons developing in culture. Images illustrate four types of neurons that were transfected at DIV 9 and visualized 1, 3 and 5 d after transfection. Neurons a and b showed relatively high level of EGFP expression 24 h after magnetofection. These neurons survived for 2.5 and 4.5 d after magnetofection, respectively. Neuron c, which showed weak and neuron d, which showed no visible EGFP expression, at day 1 developed normally for more than 5 d. **(d)** Longitudinal analysis of neuronal survival in a typical experiment. The left end of the bar is the time when neurons first displayed EGFP fluorescence; the right end indicates death of the neurons as shown by their morphology. Neurons illustrated in **c** are highlighted with color and marked using corresponding letters. Similar results were obtained in five additional experiments. Note that 13 out of 31 transfected neurons survived for more than 5 d.



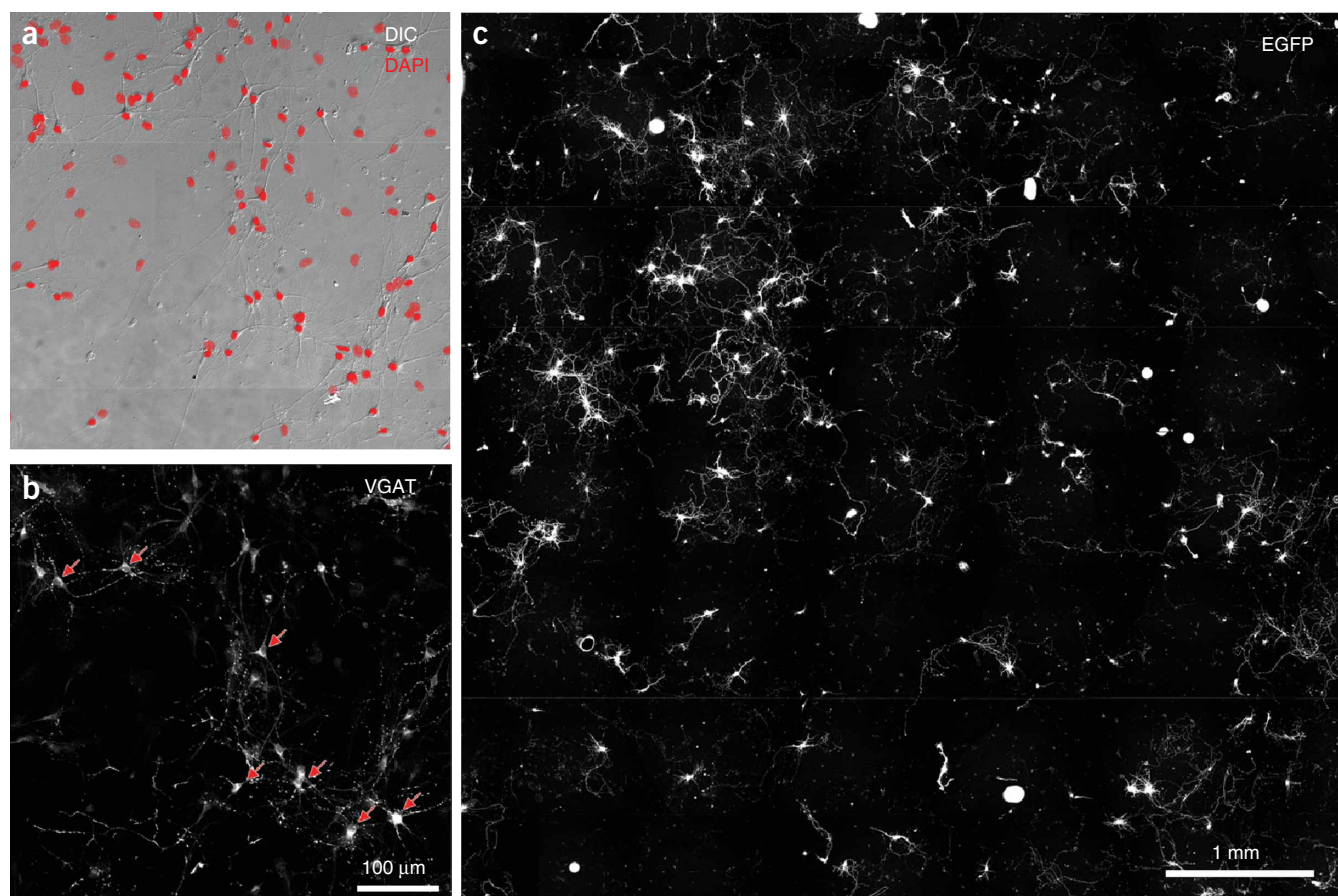


Figure 2 | Demonstration of the efficiency of magnetofection using an EGFP construct and neurons cultured according to Method A. **(a)** DAPI labeling (red) shows all cell nuclei, and DIC imaging was used to identify neurons. Approximately 20% of all cells are neurons. **(b)** The same cells shown in **a** are labeled with the marker VGAT. Cells displaying a strong somatic staining and a punctuated staining on dendrites were identified as GABAergic interneurons (red arrows). Approximately 3% of all neurons are such interneurons. **(c)** EGFP labeling demonstrates the number of magnetofected neurons. Scale bars: **(a,b)** 100 μm ; **(c)** 1 mm.

GFP (EGFP)-tagged for easy detection by fluorescence microscopy. This method is best suited for morphological or functional analysis of single cells.

Transfection of mature neurons in culture is more difficult than transfecting immature neurons. Our method allows successful magnetofection of neurons at different time points in culture, allowing to either study synaptic development in young neurons (< 10 DIV) or magnetofect mature cells (> 10 DIV), which have built up a synaptic network. Using different DNA or shRNA vectors in mixed neuron–glia cocultures, we noticed that these constructs are efficiently expressed in neurons but not astrocytes.

Seeking an optimal method of gene expression, we also tested, in parallel with magnetofection, electroporation of hippocampal cultures as well as the calcium-phosphate method of transfection, using established protocols^{17,18}. We found that out of these three approaches only magnetofection allowed efficient and highly reproducible transfection of our neuronal cultures at later stages of development (10–21 DIV). We therefore concentrated our efforts on optimizing magnetofection. A recent publication reported an improved calcium-phosphate approach that also allowed neuron transfection at later stages of development, although double-transfection was not mentioned and the focus was on GFP¹. Nevertheless, our magnetofection protocol remains an alternative, simple and powerful technique of neuronal transfection that does not require complex manipulations with different incubators, coverslip transfer, and use of blockers of *N*-methyl *D*-aspartate channels and does not deal with calcium-phosphate precipitate, a product that can potentially modify calcium balance in developing neurons and influence phosphorylation processes, leading to cell death.

Optimized neuronal culture for magnetofection

We found that optimization of the neuronal culture medium is an essential requirement for a high magnetofection rate and optimal neuronal development and survival. Key points to note are that we do not use Banker type cultures, nor glia feeder layer; instead, we use a glia–neuron coculture with defined media. Depending on the magnetofection time point, optimal results depended on appropriate cell density and medium composition, notably, the addition of B27 and the appropriate Nu-serum concentration (see **Table 1**). B27 appears to be essential for long-term neuronal survival, whereas Nu-serum allows balancing the density of neurons and astrocytes in mixed cultures. Further, the CO₂ concentration and the pH of the medium are important

parameters for achieving efficient magnetofection. The three methods presented need to be tested and adjusted, based on the individual results and experimental requirements.

Cell culture characterization and magnetofection rates

Figure 1 shows experiments investigating the efficacy of culture transfection at different stages of development and the kinetics of exogenous protein (EGFP; Clontech vector no. 6077-1) expression. To enable comparison of transfections performed at different stages, all experiments were performed using Method C and on nominally identical cultures (i.e., same neuronal densities at plating and same procedures of culture feeding). In all cultures, the first fluorescent neurons expressing GFP appeared 16 h after magnetofection. The peak of EGFP expression was reached 36 h after magnetofection (**Fig. 1a**). Afterward, the number of transfected neurons progressively decreased; however, at least 5% of neurons transfected at 9 DIV survived for more than 10 d (**Fig. 1a**). Interestingly, long-time-surviving neurons were neurons in which detectable levels of EGFP expression appeared only 48–72 h after magnetofection (**Fig. 1c,d**). Most of the neurons expressing high levels of the exogenous protein 16–24 h after transfection disappeared within 2–3 d. Taken together, this suggests that the disappearance (death) of transfected neurons was rather related to neuron exhaustion promoted by extensive protein synthesis than to toxicity of GFP itself.

The number of transfected neurons in most of the experiments performed at 5–9 DIV (Method C) ranged from 30 to 60 neurons per 14 mm coverslip with extremes of 10 and 165 transfected neurons (**Fig. 1b**). In older cultures, the number of transfected neurons decreased with culture age and ranged from 2 to 20 neurons in transfections performed at 21 DIV.

Magnetofection of 5–10 DIV neuronal cultures was extremely reliable and could be performed following any method (A, B or C) and in cultures with different neuron densities. Methods A and B allow high efficacy of transfection and short-term protein expression (see **Fig. 2**), whereas Method C is optimized for long-term expression. After establishing our techniques, we practically had no failures in neuron transfection (more than 150 magnetofections were performed by the Medina laboratory (Methods B and C), 70 transfections by the Fritschy laboratory (Methods A and B) and more than 80 transfections by the Fuhrer laboratory (Method A)).

Magnetofection of mature neurons (> 14 DIV) is more delicate and requires optimization of neuronal density and regular media exchange (Method C). Using this approach, we obtained successful transfection of 27 out of 38 cultures at 21 DIV. We noted that all failures of neuronal magnetofection at 21 DIV were related to bad culture qualities (low neuron density or neuronal death after media change), but never to the magnetofection procedure itself.

In all three methods, all our attempts to substitute the culture media, described in MATERIALS and PROCEDURE, with alternative media were negative: the cellular morphology or transfection efficacy were worse.

We have, however, obtained a similar degree of neuron transfection in all our experiments performed in 5 DIV hippocampal cultures prepared from newborn rat embryos (more than 50 magnetofections⁸) and in 5–10 DIV high-density cultures of cortical

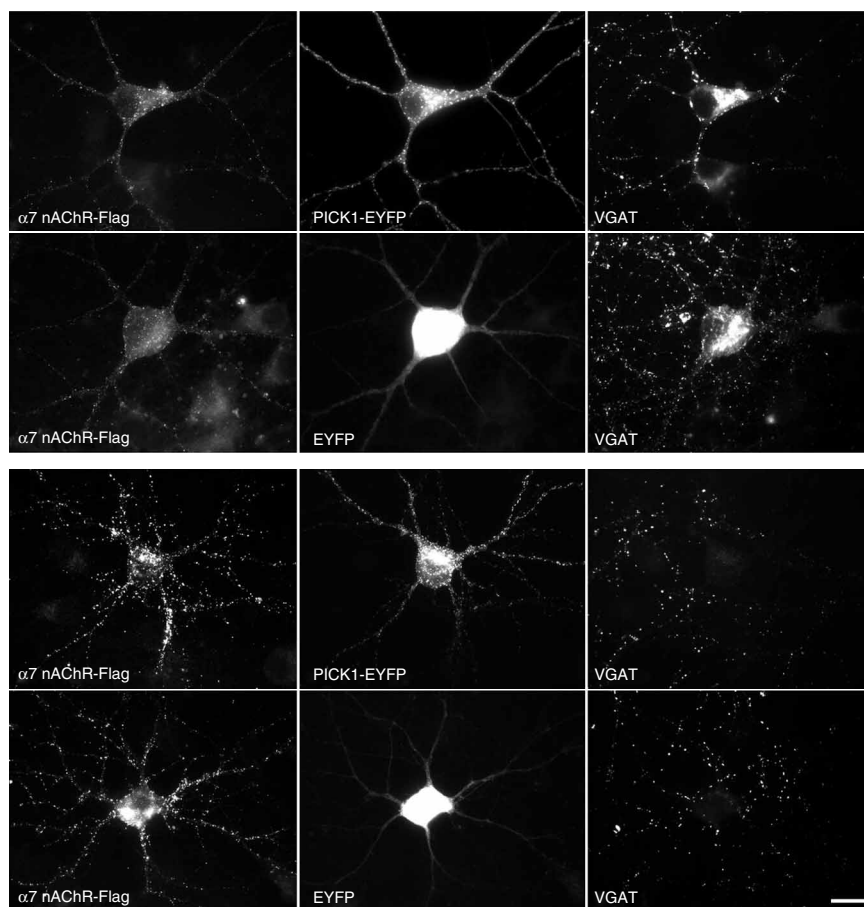
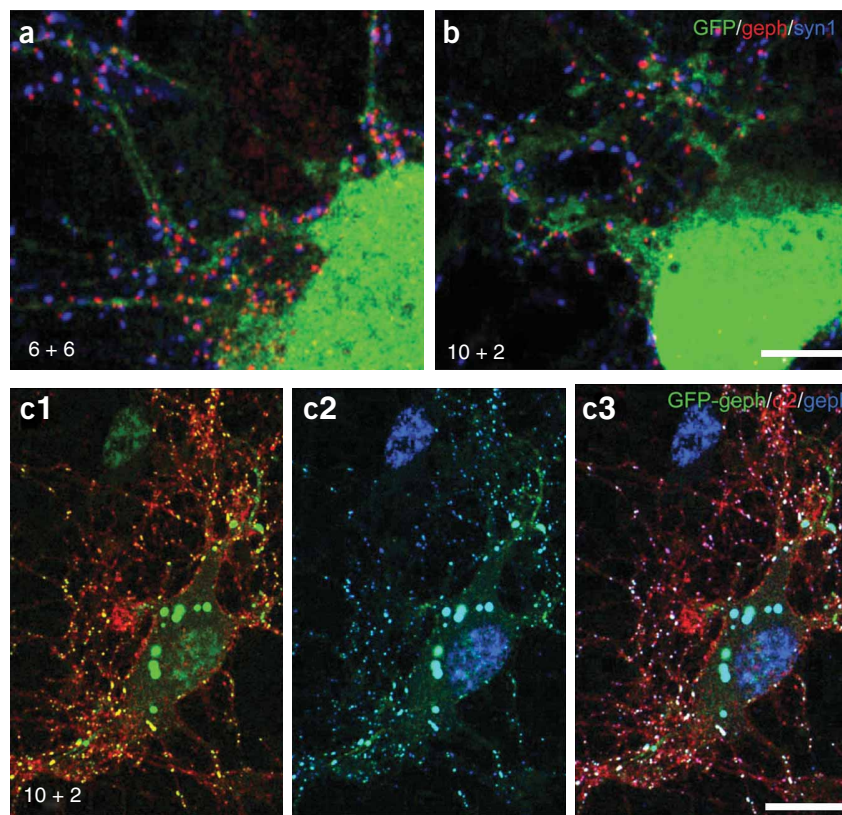


Figure 3 | Expression of PICK1 and $\alpha 7$ nAChR by magnetofection in cultured hippocampal GABAergic interneurons and pyramidal cells (Method A). Cultured hippocampal cells were transfected at 12 DIV with PICK1-EYFP or EYFP constructs and $\alpha 7$ nAChR-Flag constructs using magnetofection, labeled with anti-Flag and anti-VGAT antibody and analyzed by conventional fluorescence microscopy. The panels show examples of surface $\alpha 7$ nAChR staining (left), EYFP fluorescence (middle) and VGAT staining (right), VGAT being a marker for interneurons. This was achieved in hippocampal GABAergic interneurons (top two rows) and hippocampal pyramidal cells (bottom two rows). Neurons expressing EYFP-PICK1 or EYFP have a healthy morphology indicating that PICK1 expression *per se* did not harm these cells. $\alpha 7$ nAChR and PICK1-EYFP clusters are visible on the cell surface in both cell populations. Scale bar, 20 μ m.

Figure 4 | GFP and GFP-gephyrin expression in young and mature neurons (method B). **(a,b)** Comparison of GABAergic synapse formation onto a neuron transfected by magnetofection with an EGFP cDNA at 6 DIV **(a)** and at 10 DIV **(b)** and examined at 12 DIV (see ref. 9 for details). The different timings of transfection did not affect the formation of gephyrin clusters (red) apposed to presynaptic terminals labeled for synapsin 1 (blue). **(c)** Expression and postsynaptic clustering of GFP-gephyrin transfected by magnetofection at 10 DIV and examined at 12 DIV. Exogenous GFP-gephyrin (green) was compared to the GABA_A receptor $\alpha 2$ subunit (red, **c1**) and to endogenous gephyrin (immunostained with the monoclonal antibody mAb7a; blue, **c2**). The overlay is shown in **c3**. Note that GFP-gephyrin forms clusters containing the $\alpha 2$ subunit (yellow in **c1** and white in **c3**) and intracellular aggregates (cyan in **c2** and **c3**). It is almost completely colocalized with endogenous gephyrin, as evidenced by the lack of blue clusters on the transfected cell. Scale bars: **(a,b)** 5 μ m; **(c)** 20 μ m.



rat neurons ($n=7$). Our magnetofection method also works efficiently in hippocampal and cortical cultures of embryonic and newborn mice (data not shown).

Figure 2 illustrates the results of a representative neuronal magnetofection at 11 DIV using an EGFP vector and Method A (**Table 1**). Analysis at 12 DIV revealed that 8% of the neurons were transfected, a proportion typically achieved with this protocol. More importantly, about 2% of GABAergic interneurons, a minor subpopulation of cells in the hippocampus that can be recognized with specific neurochemical markers, were also transfected. We noticed that GABAergic interneurons are more resistant to magnetofection than pyramidal cells and we routinely obtained 3–15 transfected interneurons per 18 mm coverslip, thus enabling efficient analysis at the single-cell level (**Fig. 3**).

Recently, OZ Bioscience introduced a new improved magnetic bead-based kit (NeuroMag) for magnetofection. This novel reagent is supposed to give higher transfection efficiency, less toxicity and does not require media change after transfection.

Application 1: GFP-gephyrin expression in young and mature neurons (Method B)

We transfected cultures with either GFP cDNAs or GFP-gephyrin cDNAs at 6 or 10 DIV and examined them at 12 DIV to determine whether long-term expression of recombinant proteins affects neuronal growth and synapse formation. Gephyrin is a cytoplasmic protein selectively localized in GABAergic and glycinergic postsynaptic densities¹¹. Exogenous gephyrin was readily detected by GFP, whereas both endogenous and exogenous gephyrin could be immunostained with antibodies for selective detection. The results of these experiments showed that magnetofection at 6 DIV with either GFP or GFP-gephyrin did not interfere with neuronal growth or synapse formation (**Fig. 4a,b**). The slow development of GABAergic synapses proceeded normally under these circumstances. Furthermore, GFP-gephyrin was expressed at levels comparable to endogenous gephyrin, allowing its detection at postsynaptic sites, in combination with specific markers. This result is remarkable, as gephyrin expression appeared physiological and long-lasting and did not interfere with neural development. For transfection at 10 DIV, Method B had to be adapted: the culture medium was exchanged 24 h before transfection with the feeding medium used for Method C (see **Table 1**). The resulting morphology of the neurons was improved by this step.

Application 2: Double-magnetofection of GABAergic interneurons (Method A)

We tested whether the magnetofection method allows expression of two genes in one neuron. The proteins chosen were $\alpha 7$ nAChR, a nicotinic acetylcholine receptor found in many neurons throughout the CNS and particularly enriched in GABAergic interneurons in the hippocampus, and PICK1, a protein involved in the trafficking of glutamate receptors and enriched at glutamatergic synapses^{10,19,20}. Our method achieved expression of these two genes in pyramidal hippocampal neurons and even in a small subpool of cells, the hippocampal GABAergic interneurons. This is quite a remarkable achievement, considering how hard neurons are to transfect, and considering that only $\sim 3\%$ of all neurons in our cultures were GAD (glutamic acid decarboxylase)- or VGAT (vesicular GABA transporter)-positive, thus revealing a low density of GABAergic interneurons.

Figure 5 | Knockdown of SynGAP expression using specific RNAi (Method C). (a) Images of neurons transfected with Lentilox 3.7 vector encoding non-silencing shRNA or SynGAP shRNA. Neurons were transfected at 9 DIV. Six days after magnetofection, cultures were fixed and double-labeled with anti-SynGAP (red) and anti-MAP2 (blue) antibodies. EGFP is shown in green. Expression of SynGAP shRNA induced complete disappearance of SynGAP clusters in transfected neurons. Occasional red clusters seen on green neurons magnetofected with SynGAP shRNA belong to dendrites of non-transfected neurons overlapping with the transfected ones. These clusters constitute ~10% as compared to the total number of clusters in neurons magnetofected with non-silencing RNAi (see **b** for quantification). (b) Decrease in the density of SynGAP-positive clusters in neurons magnetofected with specific shRNA as a function of time period after magnetofection. Cluster density was normalized at each time point to mean cluster density in neurons transfected with non-silencing RNAi.

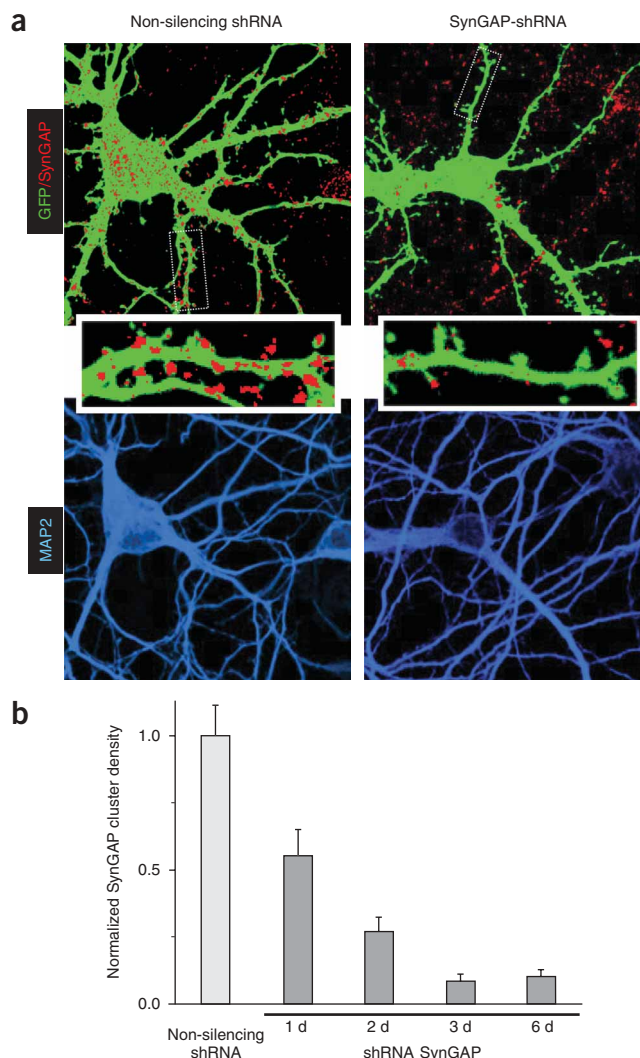


Figure 3 shows a selection of pictures demonstrating successful transgene expression after magnetofection of hippocampal GABAergic interneurons and hippocampal pyramidal cells. Double-magnetofection was performed using $\alpha 7$ nAChR-Flag, together with PICK1-EYFP or EYFP expression constructs. $\alpha 7$ nAChR surface cluster expression was detected using an anti-Flag antibody, and VGAT was used as a marker for interneurons.

Application 3: shRNA magnetofection (Method C)

This method is also suitable for gene knockdown using vectors expressing RNAi-inducing shRNAs. As an example, we illustrate data obtained after expression in neuronal cultures of the Lentilox 3.7 vector²¹ encoding shRNA against SynGAP (sequence reported in ref.22). Lentilox 3.7 vector coexpresses EGFP as a reporter gene and allows easy identification of the magnetofected neurons by fluorescence microscopy (**Fig. 5a**). Neurons magnetofected with SynGAP RNAi show a decreased level of SynGAP expression 24 h after magnetofection (**Fig. 5**). Three days after magnetofection and beyond (up to 6 d), clusters of SynGAP were not detectable anymore in all magnetofected neurons.

In conclusion, magnetofection is an inexpensive and rapid method that gives successful results for young as well as mature neurons, using different DNA or shRNA constructs, achieves long-lasting expression and even double-magnetofection of a subpopulation of cells.

ACKNOWLEDGMENTS We thank Grigory Kravinsky (Harvard Medical School, Boston) for help with preparing of shRNA coding vector. We are grateful to Corinne Sidler, Susanne Erb-Vögtli and Dubravka Göckeritz-Dujmovic (University of Zürich) for excellent technical assistance. This study was supported by grants from the Swiss National Science Foundation (to C.F. and J.-M.F.), the Swiss Foundation for Research on Muscle Diseases (to C.F.), INSERM (to C.P. and I.M.) and the French Foundation for Medical Research (to I.C.).

Published online at <http://www.natureprotocols.com>

Rights and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

- Jiang, M. & Chen, G. High Ca^{2+} -phosphate transfection efficiency in low-density neuronal cultures. *Nat. Protoc.* **1**, 695–700 (2006).
- Watanabe, S.Y. *et al.* Calcium phosphate-mediated transfection of primary cultured brain neurons using GFP expression as a marker: application for single neuron electrophysiology. *Neurosci. Res.* **33**, 71–78 (1999).
- Kim, J.B. *et al.* Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg. *J. Control. Rel.* **114**, 110–117 (2006).
- Tonges, L. *et al.* Stearilated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. *RNA* **12**, 1431–1438 (2006).

- Goetze, B., Grunewald, B., Baldassa, S. & Kiebler, M. Chemically controlled formation of a DNA/calcium phosphate coprecipitate: application for transfection of mature hippocampal neurons. *J. Neurobiol.* **60**, 517–525 (2004).
- Zeitelhofer, M. *et al.* High-efficiency transfection of mammalian neurons via nucleofection. *Nat. Protoc.* **2**, 1692–1704 (2007).
- Plank, C. *et al.* The magnetofection method: using magnetic force to enhance gene delivery. *Biol. Chem.* **384**, 737–747 (2003).
- Chudotvorova, I. *et al.* Early expression of KCC2 in rat hippocampal cultures augments expression of functional GABA synapses. *J. Physiol.* **566**, 671–679 (2005).
- Lardi-Studler, B. *et al.* Vertebrate-specific sequences in the gephyrin E-domain regulate cytosolic aggregation and postsynaptic clustering. *J. Cell Sci.* **120**, 1371–1382 (2007).
- Baer, K. *et al.* PICK1 interacts with $\alpha 7$ neuronal nicotinic acetylcholine receptors and controls their clustering. *Mol. Cell Neurosci.* **35**, 339–355 (2007).
- Sassoe-Pognetto, M. & Fritschy, J.M. Mini-review: gephyrin, a major postsynaptic protein of GABAergic synapses. *Eur. J. Neurosci.* **12**, 2205–2210 (2000).
- Kim, J.H., Liao, D., Lau, L.F. & Huganir, R.L. SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. *Neuron* **20**, 683–691 (1998).

13. Chen, H.J., Rojas-Soto, M., Oguni, A. & Kennedy, M.B. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron* **20**, 895–904 (1998).
14. Paradis, S. *et al.* An RNAi-based approach identifies molecules required for glutamatergic and GABAergic synapse development. *Neuron* **53**, 217–232 (2007).
15. Brunig, I., Scotti, E., Sidler, C. & Fritschy, J.M. Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons *in vitro*. *J. Comp. Neurol.* **443**, 43–55 (2002).
16. Brunig, I., Suter, A., Knuesel, I., Luscher, B. & Fritschy, J.M. GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABA(A) receptors and gephyrin. *J. Neurosci.* **22**, 4805–4813 (2002).
17. Xia, Z., Dudek, H., Miranti, C.K. & Greenberg, M.E. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J. Neurosci.* **16**, 5425–5436 (1996).
18. Jacquier, A. *et al.* Alsin/Rac1 signaling controls survival and growth of spinal motoneurons. *Ann. Neurol.* **60**, 105–117 (2006).
19. Kawai, H., Zago, W. & Berg, D.K. Nicotinic alpha 7 receptor clusters on hippocampal GABAergic neurons: regulation by synaptic activity and neurotrophins. *J. Neurosci.* **22**, 7903–7912 (2002).
20. Terashima, A. *et al.* Regulation of synaptic strength and AMPA receptor subunit composition by PICK1. *J. Neurosci.* **24**, 5381–5390 (2004).
21. Robinson, D.A. *et al.* A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **33**, 401–406 (2003).
22. Kim, M.J., Dunah, A.W., Wang, Y.T. & Sheng, M. Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. *Neuron* **46**, 745–760 (2005).
23. Schrader, N. *et al.* Biochemical characterization of the high affinity binding between the glycine receptor and gephyrin. *J. Biol. Chem.* **279**, 18733–18741 (2004).
24. Xia, J., Zhang, X., Staudinger, J. & Huganir, R.L. Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. *Neuron* **22**, 179–187 (1999).