Chemically Controlled Formation of a DNA/Calcium Phosphate Coprecipitate: Application for Transfection of Mature Hippocampal Neurons

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ABSTRACT: Numerous methods exist for transfecting postmitotic neurons, for example, DNA/calcium phosphate coprecipitation, cationic lipids, viruses, and physical methods such as microinjection, electroporation, and biolistics. Most methods, however, are either toxic to the cell, yield only poor transfection efficiencies, or cells have to be electroporated before plating. In this article, we present a standardized and fast transfection method using DNA/calcium phosphate coprecipitates that efficiently transfer DNA into mature, postmitotic hippocampal neurons. Shifting to CO_2-independent media with a well-defined pH allows for the tight control of the coprecipitate formation and for adjusting the transfection parameters for the individual DNA plasmid used. The two critical parameters for reproducible and efficient transfections are: the precise pH during crystal formation, and the incubation time of the cells with the coprecipitate. This improved procedure now enables biochemical approaches. By transfecting a dominant-positive Ras mutant, we activate the Erk/MAP kinase signal transduction pathway. Furthermore, using a siRNA plasmid directed against MAP2, the level of an endogenously expressed protein is down-regulated upon transfection. These two approaches demonstrate that the presented transient transfection method can now be used to address questions on a biochemical level in hippocampal neurons. © 2004 Wiley Periodicals, Inc. J Neurobiol 60: 517–525, 2004

Keywords: **hippocampal neurons; DNA/calcium phosphate coprecipitate; transfection method; dendritic spines; siRNA**

INTRODUCTION

A series of publications (for review, see Washbourne and McAllister, 2002) report on transfection of neurons in culture, including DNA/calcium-phosphate coprecipitation (Xia et al., 1996; Passafaro et al., 2003), electroporation (Teruel et al., 1999; Dityateva et al., 2003), microinjection (Shan et al., 2003), biolistics (Thomas et al., 1998), lipid carriers (Kaech et al., 1996; Ma et al., 2002), and most successfully, numerous viral vector systems (for review, see Craig, 1998). Each method, however, has its strict limitations. The viral methods, that is, Semliki Forest or Sindbis Virus, yield high efficiencies, but are time-consuming. Additionally, high security laboratory standards are often required. DNA/calcium-phosphate coprecipitates, as well as lipid-mediated methods, are usually simple and convenient, but only yield low efficiencies. A recent article indicates that nucleofection (Dityateva et al., 2003; Amaxa Biosystems, Cologne) yields high transfection efficiencies. The draw-

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back of this novel approach is threefold: first, it is very expensive; second, a large number of cells are required; and third, neurons have to be transfected before plating. The latter is a disadvantage for the use of mature neurons. Last but not least, physical methods to introduce macromolecules into primary neuronal cultures are rather inefficient. All of these technical obstacles in transfecting postmitotic neurons have caused notorious difficulties in routine expression of exogenous proteins for functional studies.

Recently, we established a DNA/calcium-phosphate-based transfection protocol for hippocampal neurons in culture yielding significant transfection efficiencies of up to 13.5% (Köhrmann et al., 1999), which allowed the detection of exogenously expressed protein by Western blot. Since then, it has become clear that the reproducibility and the transfection efficiency are strongly correlated to the cell culture experience of the experimenter. We therefore set out to determine the exact chemical parameters that influence the reproducible formation of the DNA/calcium phosphate coprecipitate as well as the optimal physiological conditions for prolonged cell survival during and after transfection. This yields significantly higher transfection efficiencies that allow us to address questions on a biochemical level in primary cultures of hippocampal neurons.

MATERIALS AND METHODS

Cell Culture

Cultured rat hippocampal neurons derived from E17 embryos were cultured as described (Goslin et al., 1998; Goetze et al., 2003).

Transfection Solutions and Media

NMEM-B27 transfection medium consisted of MEM (Life Technologies/Invitrogen, Karlsruhe, Germany) supplemented with 1 m*M* sodium pyruvate (Sigma, Deisenhofen, Germany), 15 m*M* HEPES (Invitrogen), 2 m*M* stable L-glutamine (PromoCell, Heidelberg, Germany), 1X B27 supplement (Invitrogen), and 10 m*M* D-glucose (Sigma). Transfection buffer was 2X BES-buffered saline (2X BBS): 50 m*M* BES, 1.5 m*M* Na_2HPO_4, 280 m*M* $NaCl_2$. HBSS washing buffer (pH 7.3) was: 135 m*M* $NaCl_2$, 20 m*M* HEPES, 4 m*M* KCl, 1 m*M* Na_2HPO_4, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 10 m*M* glucose. All unspecified chemicals used were from Merck (Darmstadt, Germany).

pH Adjustments of NMEM-B27 Medium and Transfection Buffer (2X BBS)

Changes in the pH of the transfection solutions greatly influence the size of the DNA/calcium phosphate coprecipitate and thus both transfection efficiencies and cell survival. Therefore, pH values of the solutions were adjusted in batches using freshly calibrated instruments. Solutions were stored at 4°C up to 6 months. To ensure reproducibility, different batches of transfection solutions were not mixed.

Plasmids

Endo-free Maxiprep kits (Qiagen, Hilden, Germany) were used for plasmid-purification. The plasmids used in this study were a kind gift from the following people: L22-EYFP expression vector and the pSUPER-MAP2 siRNA plasmid were from Dr. Paolo Macchi in the lab (P. Macchi and MAK, unpublished); the neuron-specific tubulin α1 promoter driving EGFP was from Dr. S. Kugler (Kugler et al., 2001); myc-HaRasLeu61 expression vector was from Dr. Renata Zippel (University of Milano, Italy); and Citrin green fluorescent protein (GFP) variant was from Dr. Virginie Georget (EMBL, Heidelberg, Germany). The $pGFP^2$-N was purchased from Perkin-Elmer (Rodgau-Jügesheim, Germany). The dsRed (RFP) was purchased from Clontech/BD Biosciences (Heidelberg, Germany). All plasmid-solutions were briefly centrifuged prior to use and only the supernatant was used for transfections.

Transfection Procedure

All solutions were used in 1 mL aliquots and warmed to ambient temperature. Plasmid DNA (3 μg) was slowly added to freshly diluted 250 m*M* $CaCl_2$ solution by stirring with the pipette tip, the total volume of both solutions being 60 μL. For double transfections, the plasmid DNA solutions (3 μg of DNA in total) were mixed before addition to the $CaCl_2$ solution. 2X BBS (60 μL) was added drop wise to the DNA/$CaCl_2$ solution. The reaction tube was gently shaken each time after addition of three drops of transfection buffer. Then, the solution was thoroughly mixed (not vortexed) by blowing air bubbles (5X) with the pipette into the solution and then pipetting up and down once. The transfection mixture was immediately added drop wise to the hippocampal neurons: on coverslips (cvs) in a 3 cm dish or glass bottom dishes containing 2 mL of transfection medium, followed by gentle swirling in order to mix medium and transfection mixture and to ensure a homogenous formation of the DNA/calcium phosphate coprecipitate. Alternatively, for eight cvs or neurons grown in 6 cm cell culture dishes (for biochemical experiments in Fig. 3), a total of 4 mL of transfection medium with the double amount of transfection mix was used [see Fig. 1(A)]. Cells were incubated without CO_2-supply in a humidified incubator at 36.5°C or in the microscopic chamber (see below) for the desired transfection time (see Results). Neurons on cvs were washed with prewarmed HBSS for 5–10 min until the DNA/calcium

phosphate coprecipitate was completely removed from the cells and subsequently incubated in culture medium. For the siRNA experiments, the expression time post-transfection was typically 3–4 days.

During the review process for this study, we discovered that the time of incubation of the neurons with the DNA/calcium phosphate coprecipitate could be significantly shortened by a brief centrifugation of the transfection reaction. In detail, the DNA/calcium phosphate coprecipitate is removed from the transfection media and laid onto the underlying neurons using an Eppendorf 5810 table centrifuge and special rotors (A-4-62) for 2–6 min at 200×g. This reduces the incubation time by a factor of 2.

Phalloidin Labeling and Immunostaining

After fixation with 4% paraformaldehyde for 10 min, cvs were either incubated for 3 min in Alexa-546-labeled phalloidin (Molecular Probes, Leiden, The Netherlands)/PBS solution (1:200) or overnight in HBSS/0.1% TX-100 (1:2000, for better contrast pictures) and washed 3X in PBS before mounting. Immunostaining using mouse monoclonal anti-PSD95 (Abcam/BioMol, Hamburg, Germany, dilution: 1:1000) and mouse monoclonal anti-myc (Sigma; dilution: 1: 250) was performed as described in Macchi et al. (2003).

Time-Lapse Videomicroscopy and Image Processing

The live cell imaging setup used has been described in detail (Goetze et al., 2003; Macchi et al., 2003). In brief, neurons in glass bottom dishes (Willco Wells, Amsterdam, The Netherlands) were transfected on the microscopic stage at 36.5°C. The final washing step was omitted in these experiments to avoid movement of the cells during time-lapse recordings. Images were then acquired using Metamorph 5.0 and processed using Photoshop 6.0 or higher. For simultaneous time-lapse videomicroscopy of phase contrast and fluorescence signals, pictures of each channel were acquired one after the other. The interval between a pair of recordings was 3 min.

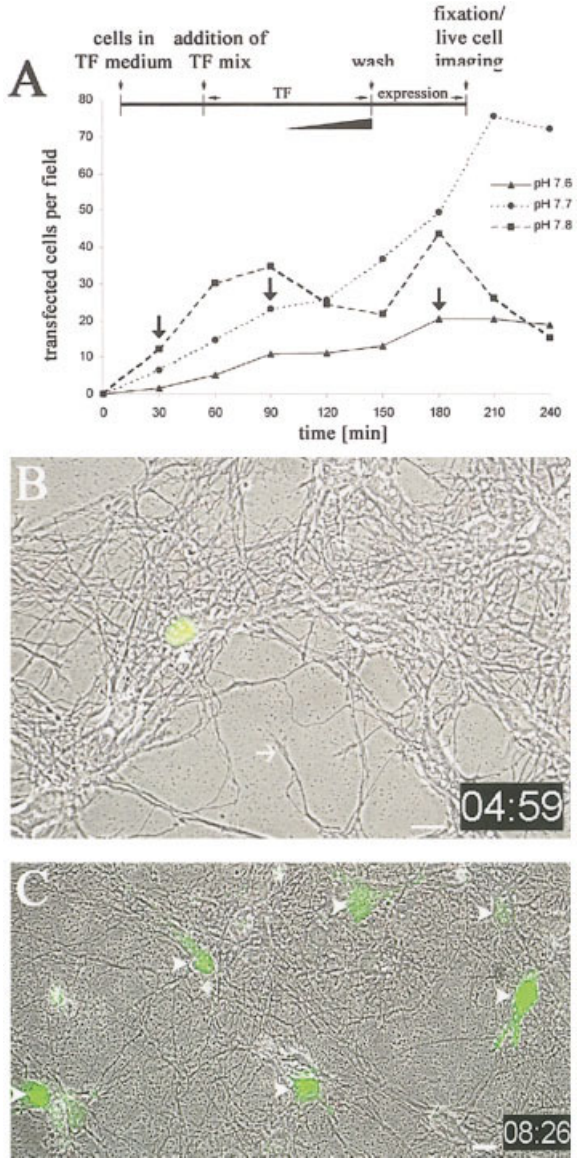

Figure 1 Chemically controlled, linear formation of the DNA/calcium phosphate coprecipitate critically determines cell viability and transfection efficiencies in mature hippocampal neurons in culture. (A) Transfection efficiencies are critically dependent on the pH of the medium and the duration of transfection. The top of the figure shows a schematic representation of the transfection (TF) procedure. Three sets of transfections at DIV 10–12 with eight coverslips each were carried out in parallel using media that varied in their pH ranging from 7.6 to 7.8. Cells were washed at the indicated time points and subsequently incubated for 12 h in culture medium. The number of cells expressing L22-EYFP was determined using a standard fluorescence microscope. Arrows in the graph indicate the time point when the DNA/calcium phosphate coprecipitate (DNA/CaPi) was first visible with a 10X objective. (B,C) Corresponding figures to Videos 1 and 2 (available online, see journal homepage): simultaneous detection of DNA/calcium phosphate coprecipitate (phase contrast) as well as the expression of the fluorescent reporter protein [EYFP for (B), GFP[2] for (C)]. Cells grown in video dishes were transfected and time-lapse videomicroscopy was performed. Six transfected neurons (see arrowheads) were found in a single microscopic field, indicating the remarkable transfection rate for this method. Both panels represent the end point of the respective video sequence. The arrow in panel (B) points at a growth cone that was not affected by the 5 h exposure to the coprecipitate, exerting normal explorative motility (see corresponding video sequence). Scale bar: 10 μm.

Data Analysis

Neurons on cvs from the same culture that expressed L22-EYFP were counted per field (20 fields per condition) using a 10X objective. The total number of neurons per field was determined by DAPI-counterstain (average #: 243 neurons per field). For example, at pH 7.7 and 210 min of exposure time, the total number of L22-EYFP-positive neurons was 75. Thus, the transfection efficiency was 31.2% in this case. For quantifications of F-actin/phalloidin labeling and anti-PSD95 immunostaining, the pictures were acquired using a 63X oil immersion objective and processed using Metamorph 5.0 in the following way: dendritic regions were manually selected, background-subtracted, and flattened, and signals were enhanced using Laplace transformation. Subsequently, images were thresholded and signals automatically counted. In total, 61 dendrites derived from three neurons for each condition with a total length of 3645 μm were analyzed.

Cell Stimulation, Preparation of Cell Extracts, and Western Blotting

Ten to thirteen DIV neurons in 6 cm dishes were transfected with an expression plasmid coding for MycHaRasLeu61 and subsequently returned for 2 h in culture medium. To reduce basal synaptic activity, neurons were then incubated for 2 h in the culture medium containing 1 μM tetrodotoxin (TTX) (Calbiochem, San Diego, CA) and finally for 1 h in Krebs-Ringer buffer also containing 1 μM TTX (Baldassa et al., 2003). Neurons were stimulated with KCl (50 m*M*, 1.5 min) or BDNF (75 ng/mL, 5 min) (Invitrogen). Cells were washed once with prewarmed Krebs-Ringer buffer and scraped in the following lysis buffer to test for Erk phosphorylation: 25 m*M* HEPES pH 7.5, 150 m*M* NaCl, 10 m*M* $MgCl_2$, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 25 m*M* NaF, 1 m*M* sodium orthovanadate, 10 m*M* β-glycerophosphate (Taylor and Shalloway, 1996). To analyze the expression level of MAP2, cells were lysed in 50 m*M* Tris-HCl pH 7.5, 100 m*M* KCl, 1 m*M* $MgCl_2$, 0.5% Triton X-100, and 1 m*M* PMSF. Both lysis buffers contained the complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 14,000 rpm for 10 min at 4°C and supernatants (15–20 μg) were subjected to Western blotting analysis using ECL (Amersham-Pharmacia, Freiburg, Germany) or for the anti-myc blot, SuperSignal West Femto (Perbio Science, Bonn, Germany) was used. Erk1/2 activity was evaluated by immunoblotting with antibodies against doubly phosphorylated Erk1 and Erk2 (Baldassa et al., 2003). The following antibodies were used in the indicated dilutions: mouse monoclonal anti-MAP2 (1:250), anti-α-tubulin (1:5000), and anti-myc (1:500; all from Sigma, Munich, Germany), anti-Erk2 (Baldassa et al., 2003; dilution 1:5000), anti-phospho-Erk (Baldassa et al., 2003; dilution 1:2000), and rabbit polyclonal anti-GFP (1:2000; Molecular Probes).

RESULTS

In order to overcome variability in transfection efficiencies and survival rates of neurons, we investigated the critical parameters that caused the varying results for the DNA/calcium phosphate coprecipitation method in the past. We realized that different DNAs and even individual DNA preparations of the same plasmid significantly contribute to variations in transfection rates. In addition, even slight changes in the pH of the transfection solutions and media greatly influence both the size of the precipitate and cell survival during transfection. We therefore chose a HEPES-buffered carbonate free transfection medium to ensure stable pH values resembling those under normal culture conditions (pH 7.5). Transfections could therefore be carried out in a CO_2-free environment without any additional cell culture equipment. The two important parameters, which critically influence the pH value, are the pH of the transfection medium (NMEM-B27) and the pH of the transfection buffer (2X BBS). The use of a CO_2-free environment thereby enables us to tightly regulate both parameters, thereby yielding chemically controlled, well-reproducible transfection conditions.

A typical experiment is outlined in Figure 1(A). Routinely, three sets of 12 days *in vitro* (DIV) or older hippocampal neurons were transfected in parallel with L22-EYFP as a reporter plasmid. This particular expression plasmid was chosen because L22-EYFP expresses very well and mainly localizes to the nucleus, facilitating the cell count. In order to experimentally determine the best transfection conditions for this plasmid, a transfection buffer (2x BBS) with a fixed pH of 7.1 was used and the optimal pH of the transfection media was titrated. In our experience, a range of pH from 7.6 to 7.8 is most suitable. Because the formation of the DNA/calcium phosphate coprecipitate takes place in the medium containing the neurons, the exposure time also critically affects transfection rates. The following time course was performed: one coverslip for each pH condition was removed from the transfection dish at the indicated time points, washed, and subsequently incubated for 12 h in culture medium. Finally, the average number of transfected cells per microscopic field was quantified for each condition.

The graph in Figure 1(A) reveals different dynamics over time for the transfection rates depending on the pH of the transfection medium used. In the case of the L22-EYFP plasmid, a pH of 7.7 for the transfection medium yields highest transfection efficiencies (31.2% in this particular experiment, see Materials and Methods). At the same time, the conditions do not

Table 1 Transfection Efficiencies for Three Different Plasmids

	10 DIV	11 DIV	12 DIV
Citrine	12.4% $n = 1076$	27.15% $n = 1068$	24.4% $n = 1041$
RFP	13.7% $n = 782$	20.6% $n = 1005$	20.3% $n = 1063$
Hras-Leu61	21.4% $n = 1288$	21.5% $n = 1805$	18.6% $n = 1727$

Neurons were transfected at the indicated day *in vitro* with the indicated plasmids and at least 700 cells or more were analyzed for protein expression. The two fluorescent proteins were detected by fluorescence microscopy, the myc-tagged Hras-Leu61 was detected by immunostaining using anti-myc antibodies.

affect cell survival rates, therefore allowing prolonged exposure time to the DNA/calcium phosphate coprecipitate of up to 210 min. An alternative to chemically control the pH of the transfection reaction is to titrate the pH of the transfection buffer (2x BBS) from 6.90 to 7.15 in 0.05 steps instead of that of the medium, which was used at pH 7.5 (data not shown). The latter is most suitable in case many different plasmids are used on a routine basis. With these two methods, transfection conditions for a new plasmid preparation can be quickly optimized by choosing typically three different pH values for either the transfection buffer or the transfection medium and varying incubation times (1.5–2 h). A clear advantage is that by this procedure, the optimal transfection conditions can be quickly determined for any given plasmid, without having to inspect optically the ideal size of the coprecipitate (Köhrmann et al., 1999).

We then went on to directly compare the individual transfection efficiencies of three example plasmids used in this study (Table 1). The data presented underline the fact that consistently high efficiencies can be obtained by our method. On the other side, we experienced significant differences in the transfection efficiencies for different plasmids during this study: see the observed range from 12.4 to 27.2% (Table 1).

Two video sequences [Fig. 1(B,C); see supplementary videos, available online at http://www.interscience.wiley.com/jpages/0022-3034/suppmat] visualize the chemically controlled, linear formation of the DNA/calcium phosphate coprecipitate by phase contrast microscopy, as well as the onset of the fluorescent reporter protein expression by fluorescence microscopy. Hippocampal neurons (10–12 DIV) were grown on glass bottom dishes, transferred to the live cell imaging setup, transfected on the microscopic stage with the indicated plasmids, and time-lapse videomicroscopy was performed. Time point zero represents the addition of the transfection mix to the cells. In Figure 1(B), the L22-EYFP fusion protein was detected shortly after 3 h upon the start of transfection, whereas another fluorescent reporter protein (codon humanized GFP[2]) took longer to become visible [Fig. 1(C)]. In the latter case**,** six transfected neurons are found in a single microscopic field using a 63X objective. Most importantly, the phase contrast video sequences clearly demonstrate that neurons remained healthy during the entire transfection procedure. Typical parameters were: fragmentation of processes including the appearance of varicosities, integrity of the nucleus, and three-dimensional structure of the cell body ("halo" around the cell body in phase contrast). This excludes the possibility that the DNA/calcium phosphate coprecipitate may be toxic to the cells. Even sensitive structures like growth cones [see arrow in Fig. 1(B)] were not affected by the 5 h exposure to the coprecipitate, because the indicated growth cone exerted normal explorative motility (see corresponding video sequence). Only upon prolonged exposure to the coprecipitate (significantly more than 5 h) did neurons start to become affected. Finally, simultaneously monitoring both transfection and expression of the fluorescent reporter (e.g., live cell imaging or alternatively, fixed cells) allows for determining the minimal time a given reporter needs in order to be detectable in neurons. This is especially relevant for plasmids with strong promoters to avoid overexpression of the chosen reporter.

We then investigated whether the transfected hippocampal neurons undergo any significant change in morphology during and after the transfection. Therefore, fully polarized, mature neurons (21 DIV) were transfected with L22-EYFP and counterstained with Alexa546-phalloidin after fixation to visualize the overall integrity of the actin cytoskeleton [Fig. 2(A), left panels]. The morphology of the cells under both conditions was also not affected based on phase contrast microscopy [Fig. 2(A), right panels]. Under these conditions, comparable patterns of phalloidin-labeled actin were detected in both transfected and untransfected cells (see inserts). To specifically visualize dendritic spines, neurons were either Alexa546-phalloidin labeled or immunostained with anti-PSD95 antibodies [Fig. 2(B)]. These experiments corroborate the fact that under these more physiological conditions even fully mature neurons greater than 14 DIV remain healthy, and their cellular architecture, for example, dendritic spines, stays intact. The neurons display an indistinguishable number of PSD95-positive synapses compared to untransfected neurons. A

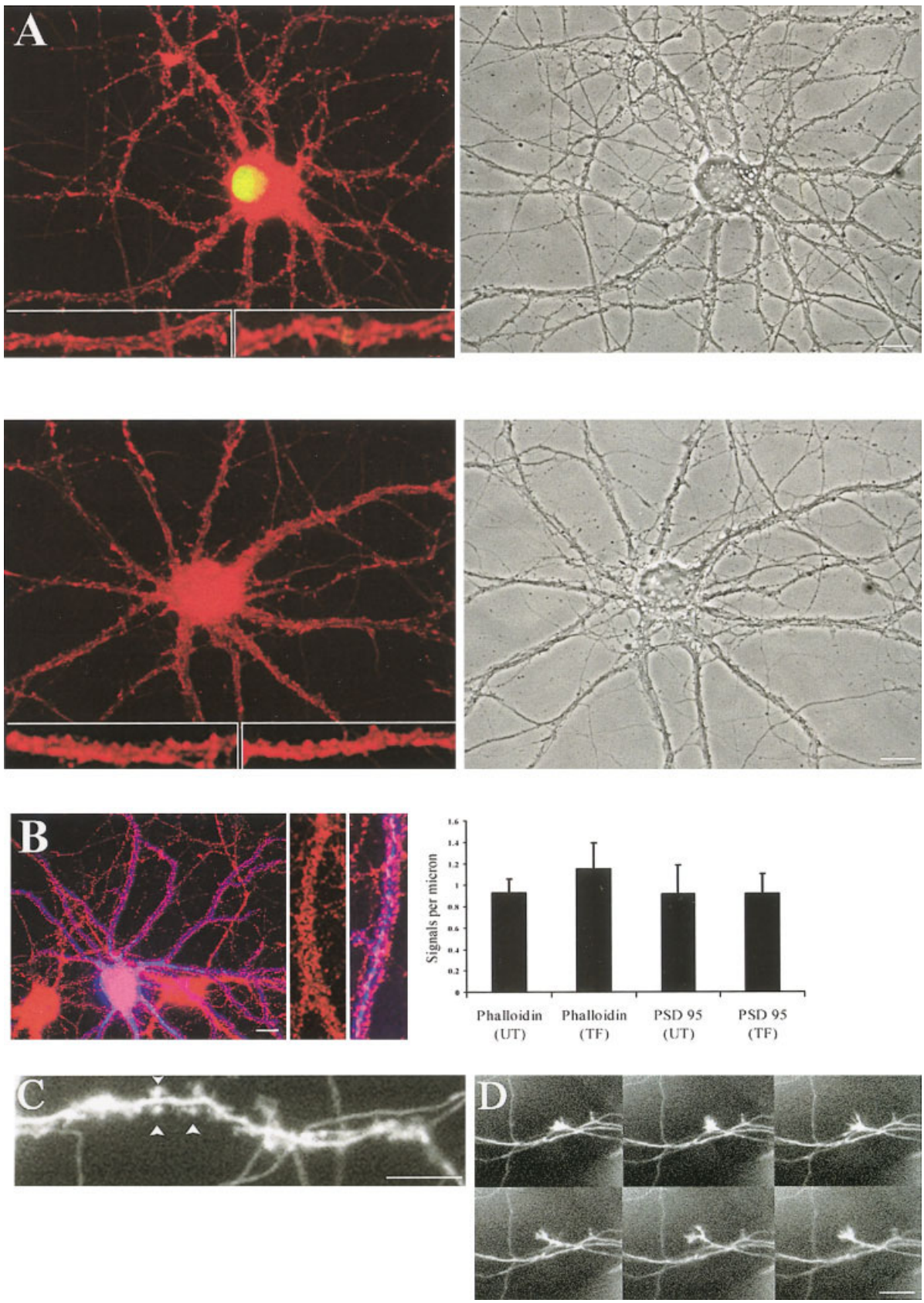
A
B
Signals per micron
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Phalloidin (UT)
Phalloidin (TF)
PSD 95 (UT)
PSD 95 (TF)
C
D

Figure 2

detailed quantification of this experiment [Fig. 2(B)] showed that the number and the localization of PSD95-positive signals on dendritic spines were basically unaffected by our transfection method. Transfection efficiencies, however, significantly drop if cells are used at this age. When we used 15 DIV neurons for transfection with a plasmid coding for the EYFP-variant Citrin, this resulted in transfection efficiencies of 9.1 and 15.8%; 21 DIV cells gave efficiencies in the range of 1% at best. A careful analysis showed that it is not due to cell death, but rather a less efficient uptake of the provided plasmids. The precise reason for this effect is not yet understood, but is under current investigation.

Cell survival and proper maturation after transfection are prerequisites for studies addressing long-term dynamics of fluorescently labeled proteins in neurons. Immature hippocampal neurons (5 DIV) were transfected with a plasmid coding for EGFP driven by the neuron-specific α1-tubulin promoter and subsequently returned into culture medium for 15 to 16 days. As shown in Figure 2(C), neurons continued to express their reporter protein at high levels and exhibited a fully developed, mature dendritic tree (data not shown). The video sequence corresponding to Figure 2(C) (see supplementary videos) shows that the observed dendritic spines (labeled by arrowheads) display normal spine motility as reported by Fischer et al., 1998. Finally, migrating growth cones [Fig. 2(D), see supplementary videos] have been observed, further underlining that hippocampal neurons transfected by this method remain healthy and intact.

In the past, biochemical approaches using neuronal cultures often failed due to the low transfection efficiencies of nonviral transfection methods. In order to demonstrate that biochemical experiments are now within range, we transiently transfected a constitutively active mutant of Ras (myc-HaRasLeu61), known to promote Erk phosphorylation levels (Joneson et al., 1996), in 10–13 DIV hippocampal neurons to interfere with the well-characterized Ras/ mitogen activated protein kinase (MAPK) pathway. Activation of MAPKs was monitored using commercially available antibodies against dually phosphorylated Erk1/2. Panel (A) of Figure 3 shows that as early as 5 h post-transfection, a significant increase of Erk phosphorylation in myc-HaRasLeu61-transfected compared to untransfected cells was detected. As a positive control, we stimulated untransfected neurons with KCl and BDNF, resulting in a marked increase of phosphorylated Erk proteins [Fig. 3(A), see also Baldassa et al., 2003]. In an independent experiment, we transiently transfected 10–12 DIV neurons with a siRNA expression plasmid to down-regulate protein expression using the newly developed technology of plasmid driven gene silencing (Brummelkamp et al., 2002). Neurons were transfected with the pSUPER MAP2 siRNA expression plasmid designed to specifically down-regulate MAP2 mRNA in combination with the tracer plasmid Citrin or with Citrin alone. Post-transfection cells (72–96 h) were scraped and lysates subjected to Western blotting. Figure 3(B) demonstrates that this transfection method is well suited to promote a strong reduction in MAP2 protein expression (see Krichevsky and Kosik, 2002). Cells transfected with Citrin alone showed similar levels of MAP2 compared to untransfected cells. These two experiments clearly demonstrate that it is feasible to address distinct biochemical questions using transfected hippocampal neurons in culture.

In neurons, it is also feasible to perform double transfections with constructs coding for fluorescent

Figure 2 Transient transfection does not alter the morphology of mature hippocampal neurons in culture. (A) Comparison of F-actin staining of untransfected and transfected 21 DIV neurons. The inserts show magnifications of two dendrites. The upper panel shows a neuron that has been transfected at 21 DIV with a plasmid coding for L22-EYFP fusion protein and fixed after 12 h of expression. The lower panel shows a comparable, untransfected cell. The actin cytoskeleton of both neurons was counter-stained with Alexa 546-labeled phalloidin. (B) Comparison of F-actin staining and anti-PSD95 immunostaining of untransfected and transfected 22 DIV neurons (left panels). The right panel shows the quantitative analysis of this experiment. F-actin/phalloidin and postsynaptic density protein of 95 kDa (PSD95) signals were counted and are displayed as signals/μm length of dendrites. (C) High magnification of a spiny dendrite of a transfected hippocampal neuron at 21 DIV. Five DIV neurons were transfected with a plasmid that codes for EGFP under the control of the neuron-specific α1-tubulin promoter, and live cell imaging was performed at 21 DIV. Arrowheads indicate motile dendritic spines. This is best seen in the corresponding Video 3 (see supplementary videos). (D) Corresponding panels to a video sequence (Video 4, see supplementary videos) of a motile growth cone found in a representative field of transfected hippocampal neurons in culture indicating that cells are viable and healthy 15 days after transfection. The total length of this video sequence was 13:34 min. Scale bar: 10 μm.

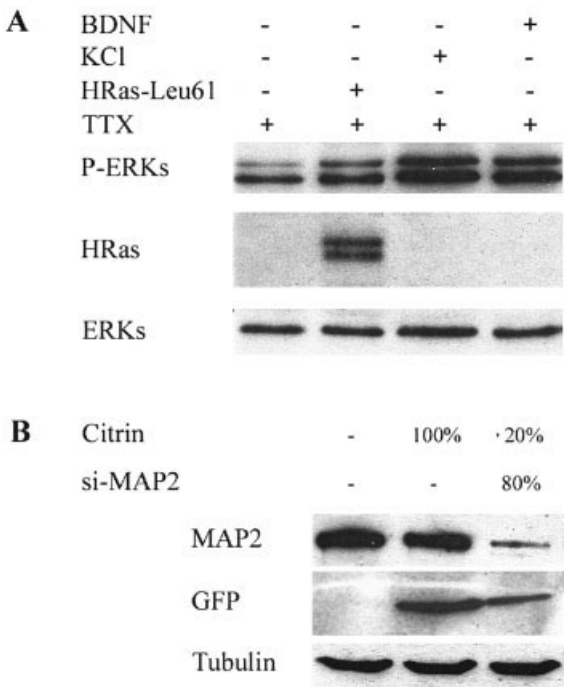

Figure 3 Biochemical interference with endogenous signaling mechanisms and protein expression by transiently transfecting mature hippocampal neurons. (A) The expression of a constitutively active Ras mutant resulted in elevated levels of extracellular signal-regulated kinase (ERK) phosphorylation. Twelve DIV rat hippocampal neurons were transfected with a plasmid encoding for myc-HA-RasL61 or mock-treated. Cells were treated for 3 h with 1 μM TTX to reduce spontaneous synaptic activity and scraped 5 h post-transfection. Lysates were analyzed by Western blotting using anti-phospho Erk (p-Erk), anti-Erk2 (Erk2), and anti-myc antibodies. The expression of myc-Ha-RasL61 was independently confirmed by immunodecoration with anti Ha-Ras antibodies (data not shown). (B) Expression of siRNAs directed against MAP2 mRNA yielded significantly reduced levels of MAP2 protein in transiently transfected hippocampal neurons. Ten DIV neurons were transfected with a combination of plasmids encoding for Citrin and pSUPER MAP2 (ratio 20:80%), Citrin alone (100%), or left untransfected. Three days post-transfection, cells were scraped and lysates processed by Western blotting using anti-MAP2, anti-GFP, and anti-α-tubulin antibodies, to warrant equal loading. Representative examples of at least three independent experiments are shown.

proteins to trace plasmids, for example, the siMAP2 plasmid. We quantified the cotransfection efficiency for one pair of plasmids, for example, Staufen1-ECFP and L22-dsRed, and reproducibly got more than 90% of the transfected neurons expressing both proteins under the chosen experimental conditions. Furthermore, we would like to stress the fact that this method is not at all restricted to hippocampal neurons. When subtle adjustments to the presented protocol were applied, various cell lines [i.e., baby hamster kidney fibroblasts (BHK), COS, and HeLa] and even primary glial cells (a detailed protocol is available upon request) were also successfully transfected (data not shown), making this method an alternative equivalent to commercial transfection reagents.

Taken together, the presented transfection method represents a significant improvement compared to previous approaches in several ways (i.e., Köhrmann et al., 1999). Firstly, the formation of the DNA/calcium phosphate coprecipitate is now well controlled and linear in respect to the growth of the crystals. Secondly, the method specifically adapted to hippocampal neurons offers a standardized procedure to determine the optimal transfection conditions for each plasmid DNA. Thirdly, due to the more physiological conditions during transfection, cells are significantly less stressed and remain healthy for prolonged times. Carefully controlling these identified parameters, especially the less basic pH values during transfection compared to Köhrmann et al. (1999), now favors high and consistent transfection rates. Fourthly, we think that this method is advantageous for mature neurons over electroporation and nucleofection (Amaxa Biosystems) because they can be conveniently transfected at the desired age, many more different transfections can be performed, and much less cells are lost during treatment. In conclusion, our improved transfection procedure is especially suitable for studies investigating the earliest visualization of a chosen reporter protein in living, postmitotic, and sensitive neurons in dissociated culture without causing any possible side effect due to overexpression. Most importantly, however, this transfection method can now be used in order to address biochemical questions using hippocampal neurons in culture.

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