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Letter to Neuroscience

NON-CLASSICAL NUCLEAR LOCALIZATION SIGNAL PEPTIDES FOR HIGH EFFICIENCY LIPOFECTION OF PRIMARY NEURONS AND NEURONAL CELL LINES

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Gene transfer into CNS is critical for potential therapeutic applications as well as for the study of the genetic basis of neural development and nerve function. Unfortunately, lipid-based gene transfer to CNS cells is extremely inefficient since the nucleus of these post-mitotic cells presents a significant barrier to transfection. We report the development of a simple and highly efficient lipofection method for primary embryonic rat hippocampal neurons (up to 25% transfection) that exploits the M9 sequence of the non-classical nuclear localization signal of heterogeneous nuclear ribonucleoprotein A1 for targeting β_2-karyopherin (transportin-1). M9-assistant lipofection resulted in 20–100-fold enhancement of transfection over lipofection alone for embryonic-derived retinal ganglion cells, rat pheochromocytoma (PC12) cells, embryonic rat ventral mesencephalon neurons, as well as the clinically relevant human NT2 cells or retinoic acid-differentiated NT2 neurons. This technique can facilitate the implementation of promoter construct experiments in post-mitotic cells, stable transformant generation, and dominant-negative mutant expression techniques in CNS cells.

Lipid-mediated gene delivery is the most widely used transfection method in genomic and cell biologic research in laboratories. Unfortunately, post-mitotic primary neurons are extremely resistant to lipofection. For example, Wang and coworkers reported less than 2.5% transfection efficiency of primary glial and neuronal cells by using 1,2-bis(oleoyloxy)-3-(trimethylammonio)-propane (DOTAP)/dioleoylphosphatidylethanolamine (DOPE) (Wang et al., 2000), consistent with the 3% transfection of rat hippocampal neurons obtained with DOTAP found by Kaech et al. (1996). Wiesenhofer and Humpel (2000) had similar findings with commercial lipids such as Fugene where the transfection efficiency was only 2.4% after a regimen of three separate 6-h lipofection episodes over 3 consecutive days. We hypothesized that nuclear import was rate limiting during lipofection of non-dividing primary neurons.

The non-classical nuclear localization signal (NLS) of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 termed M9 (Michael et al., 1995) was employed in this study as previously described (Subramanian et al., 1999) using G_{265}–Y_{395} of hnRNP A1 linked to a cationic peptide derived from a scrambled variant of the SV40 large T antigen NLS peptide (KCRGKVPGKYGKG). We lipofected rat hippocampal primary neurons with commercially available lipofection reagents: Fugene (Roche), Lipofectin (Life Technology), Lipofectamine (Life Technology) or Geneporter (Gene Therapy Systems), following the manufacturers' instructions. Two days post-transfection, cells were imaged by epifluorescence and light microscopy or sorted by flow cytometry for determination of EGFP transfection. We found that all four commercial reagents tested gave less than 0.5% transfection of rat hippocampal neurons, typically no or a few EGFP-positive neurons per dish. With Lipofectamine (DOSPA/DOPE), which was comparable to the other reagents, we detected a few transfected glia cells and averaged less than 0.1 transfected neuron per mm^2 (Fig. 1A). Longer lipofection times beyond 2 h resulted in neuron cytotoxicity. Since lipofection is greatly

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Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DOTAP, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane; hnRNP, heterogeneous nuclear ribonucleoprotein; NLS, nuclear localization signal.

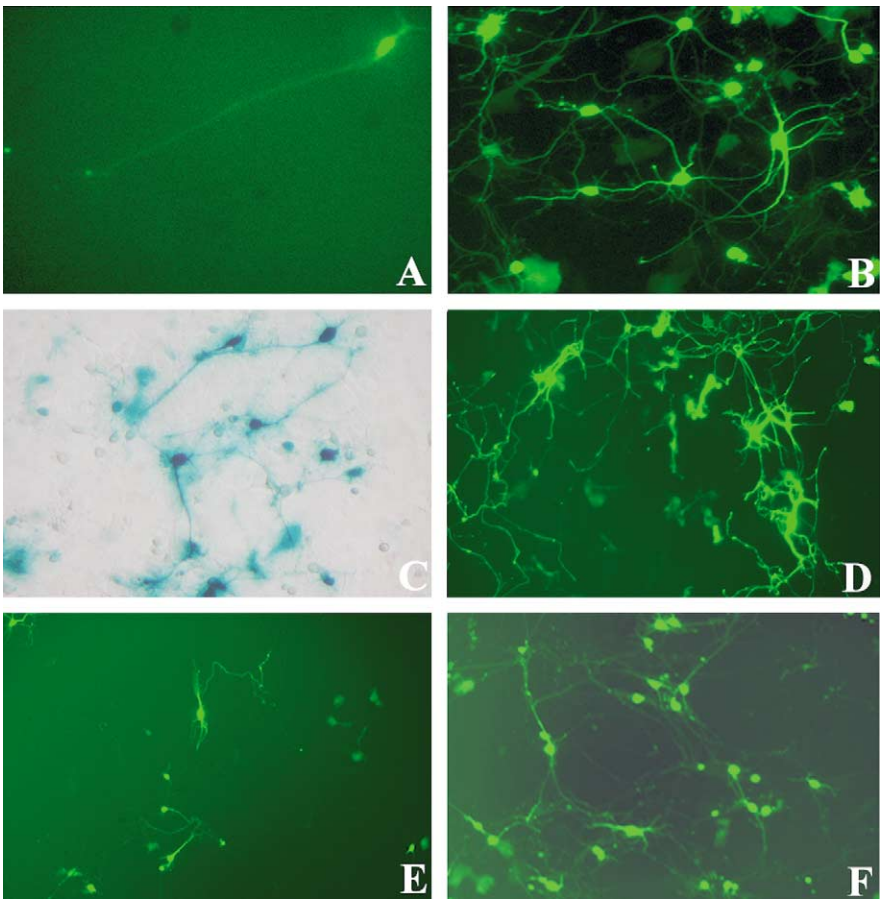

Fig. 1. Enhancement of lipofection of primary neurons using the non-classical NLS, M9 precomplexed to plasmid. Primary rat hippocampal neurons were lipofected with Lipofectamine and pCMV-EGFP (3 μg lipid per μg plasmid) in the absence (A) or presence of the M9 peptide conjugate (B) and imaged at 2 days post-lipofection. M9-assisted lipofection of pCMVβgal also resulted in high efficiency transfection as seen in Xgal-stained neurons at 2 days post-lipofection (C). No cytotoxicity due to lipofection protocol or EGFP expression was noted at 10 days post-lipofection (D). Transfection of neurons with SV40 T-antigen classical NLS (E) showed lower efficiency than with non-classical NLS, M9 (F).

enhanced by cellular division when the nuclear barrier breaks down (Tseng et al., 1999), NLSs may enhance gene transfer to non-dividing primary neurons. We precomplexed 1 μg of plasmid with 50 μg of the M9 peptide conjugate in 100 μl OPTIMEM at room temperature for 15 min before mixing with Lipofectamine (1 μl at 2 μg/μl). M9-assisted lipofection resulted in >50-fold increase to 25% transfection using pCMV-EGFP (4.7 kb) or pCMVβgal (7.2 kb) with 30–50 EGFP-positive or β-galactosidase (βgal)-positive neurons per mm^2 ($n=3$) (Fig. 1B, C). Transfected neurons were healthy with elongated neurites maintained for over 10 days (Fig. 1D). Sufficient metabolism allowed the EGFP expression to be detected within 4 h post-lipofection (data not shown). For direct comparison of M9-assisted lipofection to the classical NLS, we also lipofected 1 μg plasmid precomplexed with SV40 large T antigen NLS peptide (CGYGPKKKRKVGG, 5–50 μg). The SV40 NLS-assisted lipofection yielded few transfected neurons with optimal results at 30:1 weight ratio of peptide to plasmid (Fig. 1E), considerably less efficient than that observed with M9-assisted lipofection (Fig. 1F). This experiment demonstrated that the non-classical NLS is more potent than the classical NLS for nuclear targeting in post-mitotic neurons.

To determine if M9-assisted lipofection worked with other primary neurons or neuronal cell lines, we lipofected chicken embryonic (6 days) retinal ganglion cells, PC12 cells, undifferentiated human embryonal carcinoma cell line NTera2 (NT2), retinoic acid-differentiated post-mitotic NT2 neuron cells (NT2N) (Hartley et al., 1999), and rat ventral mesencephalon neuron cultures. The lipofection of embryonic chicken retinal ganglion cells with Lipofectamine resulted in $<0.2\%$ transfection efficiency (Fig. 2A), but the M9-assisted lipofection resulted in $\sim$100-fold increase to about 20% transfected cells in three separate experiments (Fig. 2B). The PC12 transfection efficiency with Lipofectamine was $<5\%$ (Fig. 2C) and the M9-assistant transfection elevated the efficiency to 35–40% (Fig. 2D) as determined by flow cytometry. Similarly, the NT2 transfection efficiency with Lipofectamine was $<0.5\%$ (Fig. 2E) and the M9-assistant transfection caused a $\sim$40-fold increase in transfection efficiency to 20% (Fig. 2F) as determined by flow cytometry. The lipofection efficiency of differentiated NT2 cells was extremely low with all four commercial reagents

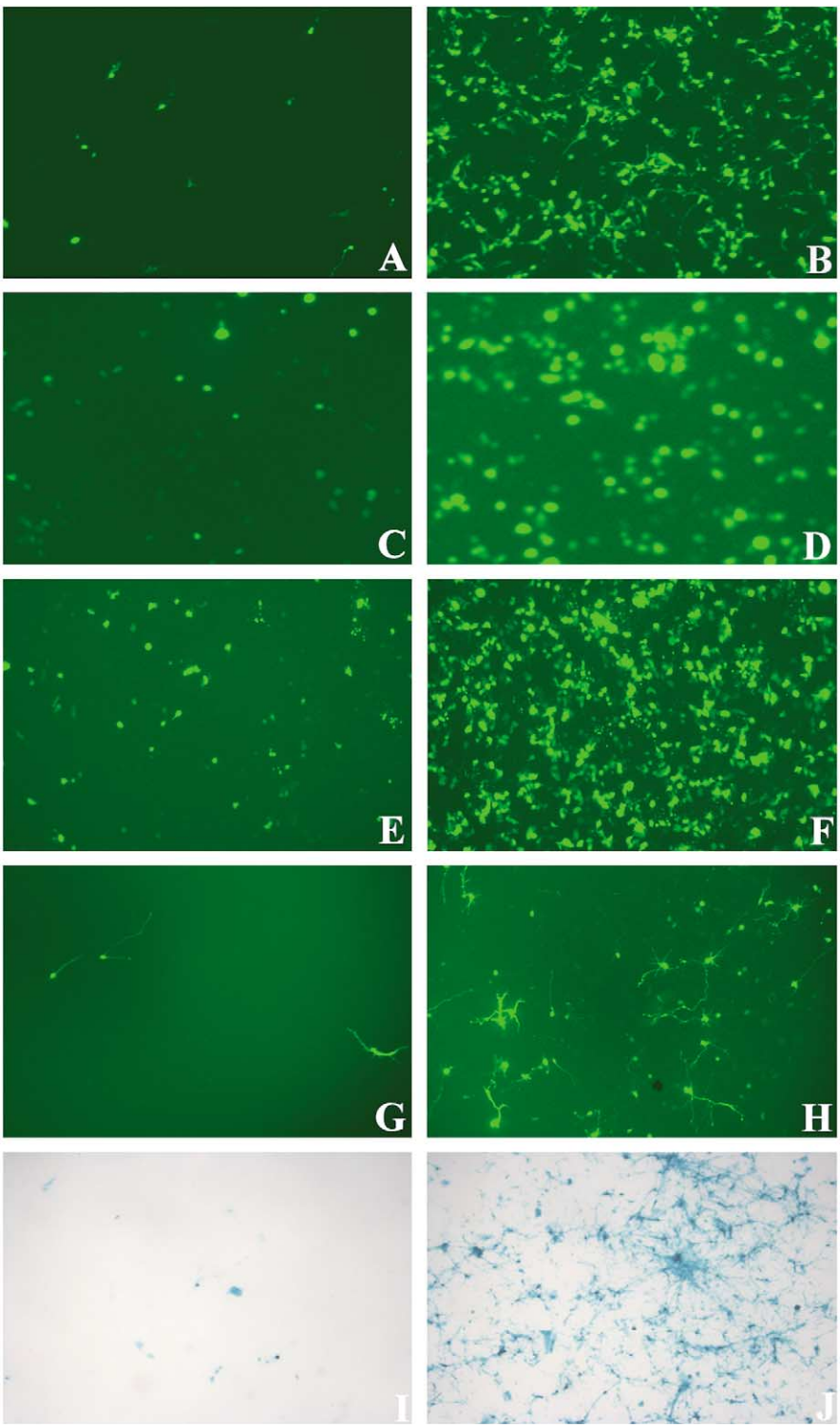

Fig. 2. EGFP or βgal expressions after lipofection of chicken embryonic retinal ganglion cells (A, B), PC12 cells (C, D), NT2 (E, F), NT2 neurons (G, H), and ventral mesencephalon cultures (I, J) with Lipofectamine and pCMV-EGFP or pCMV-lacZ in the absence (A, C, E, G, I) or presence of M9 peptide (B, D, F, H, J).

we tested (Fig. 2G), and the M9-assisted transfection resulted in an increase of ∼10-fold (Fig. 2H). Similarly, lipofection of rat ventral mesencephalon neuron cultures was extremely inefficient with only a few βgal-positive neurons per dish (Fig. 2I), but was enhanced ∼20-fold with M9-assisted lipofection (Fig. 2J).

In contrast to lipofection, viral vectors such as adenovirus or baculovirus have a high transduction efficiency in nerve cells but require significant investment in vector development and caution in terms of local immune response (Barkats et al., 1998; Sarkis et al., 2000). In the laboratory, calcium phosphate precipitation of plas-

mid results in transfection efficiencies ranging from 1 to 5% transfection in nerve cells as detected with plasmids driving expression of green fluorescent protein or βgal (Xia et al., 1996; Tabuchi et al., 1998; Watanabe et al., 1999; Jaworski et al., 2000). Unfortunately, calcium precipitation is extremely sensitive to minuscule variations in pH, ionic strength, temperature and buffer age, all of which cause poor repeatability among different laboratory members and different laboratories. In contrast, ballistic delivery provides suitable transfection up to 10% with neurons (Wellmann et al., 1999) but specialized equipment and lethality issues are substantial. Micropipette electroporation has recently been shown effective for single cell gene delivery *in vivo* (Haas et al., 2001) but is not designed as a therapeutic or large population approach. Electroporation techniques can produce high levels of transfection in proliferating PC12 cells that survive (Espinet et al., 2000), but the overall PC12 transfection efficiency is only about 10% (Akamatsu et al., 1999).

We have demonstrated a simple protocol to lipofect more than 20% of primary rat hippocampal neurons. These gains are the result of non-classical NLS, M9 sequence that yielded gains in expression superior to those found with the classical NLS. The efficient lipofection of large numbers of neurons or cultivated cells such as PC12, NT2, NT2N enables plasmid-based protocols as well as facilitates the creation of potentially therapeutic transgenic neurons for implantation without the immunological consequences typical of viral transduction for transgene expression.

EXPERIMENTAL PROCEDURES

Tissue culture

The rat hippocampal primary neurons were freshly prepared every week by hippocampal dissection. After the pregnant Sprague–Dawley rat was killed under CO_2 anesthetization, the embryos were transferred in a 60-mm Petri dish with HBS 0/0 (HEPES-buffered saline without Ca^{2+} and Mg^{2+}). The C-shaped hippocampus was exposed by peeling back one hemisphere of cortex and then cut off in HBS 0/0. Hippocampi were trypsinized for 20 min at 37°C, in a 5% CO_2 incubator, and the cells were then thoroughly broken up by pipetting with complete media and then plated on poly-lysine-coated 12-mm cover slips with a density of 40×10^5 cells/ml. Ventral mesencephalon neurons were prepared freshly after anesthetization of pregnant Sprague–Dawley rats with CO_2 on embryonic day 14. A 1-mm×1-mm section of tissue was dissected from the ventral mesencephalon of embryos in L-15 medium containing 10% fetal bovine serum (FBS). Pooled tissue sections were washed in medium without serum and then digested in 10 U/ml papain (Worthington) for 15 min at 37°C. Cells were dissociated, counted and then plated at a density of 75 000 cells/cm^2 in poly-lysine/matrigel-coated eight-well glass chamber slides (Nunc) with medium of 1:1 DME/F12 containing 15 mM HEPES and 10% FBS (Gibco). Cultures were treated with 1 mM cytosine arabinoside, 1 mM 5-fluorodeoxyuridine and 1 mM uridine between days 5 and 7 of culture, to reduce the number of non-neuronal cells. Transfection was performed on day 8 or 9 of culture. Chicken embryonic (6 days) retinal ganglion cells were kindly supplied by Drs. J. Li and J. Raper (University of Pennsylvania). The chicken 6-day embryonic ganglion cells were freshly dissected and plated, then lipofected on the second day. Rat pheochromocytoma (PC12) cells were kindly provided by Dr. R. Pittman (University of Pennsylvania) and NT2 and NT2N were cultivated as previously described (Hartley et al., 1999).

Lipofection and transgenes' expression

Fugene, Lipofectin, Lipofectamine, and Geneporter lipofection were performed by following the manufacturers' instructions. One μg of pCMV-EGFP (Clontech) was diluted in 100 μl OPTIMEM (Gibco-BRL) in one tube. Increasing doses of the various transfection reagents (0.5–3 μl Fugene; 1–3 μl Lipofectin; 1–4 μl Lipofectamine; 2–6 μl Geneporter) were tested by diluting in 100 μl of the same media in a second tube. The plasmid and transfection reagents were then mixed and incubated at room temperature based on the manufacturers' instructions. Cells were overlaid with transfection solutions (200 μl), centrifuged (100×*g*, 3 min), and incubated for 2 h before replacement with growth media. M9-assisted lipofection was performed in a similar way except that 1 μg of plasmid was precomplexed with 50 μg of the M9 peptide conjugate in 100 μl OPTIMEM and incubated for 15 min at room temperature before mixing with Lipofectamine. Following transfection, EGFP-positive cells were detected by fluorescence microscopy using a Leica fluorescence microscope equipped with the Openlab imaging program. For quantitative determinations of transfection efficiency, fluorescent cells were counted in more than three different views and followed with detection by fluorescence-activated cell sorting (FACS) using a FACScan flow cytometer (Becton-Dickinson) and CellQuest software. The pictures of βgal expression were directly taken after Xgal staining.

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