Comparative Evaluation

Different Influences of Lipofection and Electrotransfection on *In Vitro* Gene Delivery to Primary Cultured Cortex Neurons

Xue-Si Zhang, MD, PhD[1,2], Jing Huang, MD, PhD[2], Cong-Qing Zhan, MD, PhD[2], Jing Chen, MD, PhD[2], Tao Li, MD, PhD[1], Alan D Kaye, MD, PhD[3], Sheng-Xi Wu, MD, PhD[2], and Lan Xiao, MD, PhD[1]

From: [1]Department of Histology and Embryology, Chongqing Key Laboratory of Neurobiology, Third Military Medical University, Chongqing, China; [2]Department of Neurobiology, The Fourth Military Medical University, Xi'an 710032, PR China; [3]Departments of Anesthesiology and Pharmacology, Louisiana State University School of Medicine, New Orleans, Louisiana 70112

Address Correspondence:
Prof. Lan Xiao
Department of Histology and Embryology, Faculty of Basic Medicine
Chongqing Key Laboratory of Neurobiology
Third Military Medical University, Chongqing, China
E-mail: xiaolan35@hotmail.com

Disclaimer: Xue-Si Zhang and Jing Huang contributed equally to this work. There was no external funding in the preparation of this manuscript. Conflict of interest: Each author certifies that he or she, or a member of his or her immediate family, has no commercial association (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted manuscript.

Manuscript received: 08-09-2015
Revised manuscript received: 09-08-2015
Accepted for publication: 09-22-2015

Free full manuscript: www.painphysicianjournal.com

Background: Many pain states are linked to central nervous system (CNS) diseases involving the dysfunction of dendritic arborization, making restoration a promising therapeutic strategy. Transfection of primary cortex neurons offers the possibility to study mechanisms which are important for the restoration of proper arborization. Its progress is, however, limited at present due to the lack of suitable gene transfer techniques.

Objective: To obtain better insight into the transfection potential of currently used techniques, 2 non-viral transfection methods, lipofection and gene electrotransfer (GET), were compared.

Study Design: This is a comparison study performed on cultured cells.

Methods: The transfection efficiency and neuronal viability, as well as the neuronal dendritic arborization after lipofection or GET, were compared. Primary cultured cortex neurons were transfected with the pEGFP-N1 plasmid, either using Lipofectamine 2000 (2, 3, or 4μL) or with electroporation, with our previously optimized protocol (200V/25 ms).

Results: Transfection efficiency and cell viability were inversely proportional for lipofection. The appropriate ratio of Lipofectamine and plasmid DNA provides optimal conditions for lipofection. Although GET offered higher transfection efficiency, it could not induce complex dendritic arborization, which made it unsuitable for in vitro gene transfer into cortex neurons.

Limitations: Limitations include species variability and translational applicability for CNS diseases and pain states related to potential toxicity.

Conclusions: Based on these findings, lipofection might be advantageous for in vitro application to primary cultured cortex neurons. Pain states, stress mediated pathogenesis, and certain CNS diseases might potentially utilize this important technique in the future as a therapeutic modality.

Key words: Lipofection, gene electrotransfer, CNS diseases, pain states, dendritic arborization, transfection of primary cortex neurons

Pain Physician 2016; 19:189-196

Cortex neurons, especially pyramidal neurons, play important roles in the normal function of the central nervous system (CNS) by forming effective synapses via extended dendrites (dendritic arborization) (1). Many pain states and stress are linked to CNS diseases involving the dysfunction of dendritic arborization, making restoration a promising therapeutic strategy. Primary cultured cortex neurons

are a well-characterized model for studying molecular mechanisms which regulate dendritic arborization and spine formation (2,3) for future applications of gene therapy.

Successful gene therapy depends on efficient insertion of functional therapeutic genes into target cells via viral, chemical, or physical techniques without causing injury (4,5). Viral vectors are considered to be the most efficient delivery system, but with the risk of insertion mutagenesis and immunological responses (6,7). Lipofection has proven to be the most successful chemical method (4), as it offers high transfection efficiency and high levels of transgene expression in various mammalian cell types (5), including rat cortical primary cultures (8). However, possible cytotoxicity and immunogenicity induction limit its *in vivo* use (6). Thus, physical methods with high efficiency are the most promising in this field.

Gene electrotransfer (GET) is the most developed and widely used physical method for in vitro (7) and *in vivo* (9,10) gene transfer. GET combines the use of plasmid DNA (pDNA) and the local application of electric pulses. The external electric field induces a transmembrane potential at the plasma membrane (11). When the induced transmembrane potential reaches threshold voltage, membrane permeability is increased (electroporation), thereby allowing transport of molecules and macromolecules into the cells. GET is a multistep process where electroporation enables the transfer of pDNA and short RNAs (12,13). In vitro GET of primary and stem cells has shown promising potential for clinical applications (14) and has been previously applied in the spinal cord for analgesia (15). Its transfection efficiency and influence on dendritic arborization in primary cultured neurons have not been clearly elucidated. Furthermore, both *in vivo* and *in vitro* transductions of neurons are typically inefficient, most likely because the extracellular matrix (ECM) forming dense perineural nets (PNNs) around neurons physically block gene access to the cell surface (16).

Nucleofection is a modified form of GET, which uses a series of high voltage pulses that enable plasmids to directly enter the nucleus and tends to result in higher transfection rates and better cell survival than that afforded by conventional GET (17). However, nucleofection is relatively expensive and the method of choice when high transfection efficiencies are essential (17). However, in the current study, we selected conventional GET based on the budget consideration and the focus of neuronal morphology as well.

Therefore, in the present investigation, an evaluation of the potential of current non-viral gene transfer techniques was evaluated. A comparison of the transfection efficiency, neuronal viability, and dendritic arborization of lipofection and GET for primary cultured cortex neurons revealed lipofection superiority over GET in efficiency of transfer genes to cultured neurons, offering a promising tool to investigate the underlying mechanisms of CNS diseases and many pain-related processes in the future.

Methods

Animals

Female pregnant C57BL/6 mice (10 – 12 weeks) were housed in a temperature-controlled environment on a 12-hour light/dark cycle with access to food and water ad libitum. All experimental procedures received prior approval from the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, China) and the ethical guidelines for animal studies.

Preparation of Mouse Primary Cultured Cortex Neurons

A single newborn (P0.5) C57Bl/6J mouse was sacrificed by decapitation, the skull was opened and the brain removed. After removal of the meninges from the whole brain, the medial neocortex regions were gently taken out and triturated 8-fold with a fire-polished Pasteur pipette with a wide opening to dissociate larger aggregates. After allowing the aggregates to sediment, the supernatant was removed and transferred to neurobasal medium containing 0.25% trypsin. This procedure was repeated twice with Pasteur pipettes of subsequently smaller opening sizes.

Cell pellets were gently re-suspended with a Pasteur pipette and 200 µL of the suspension containing 3×10^5 cells were seeded onto 35 mm poly-l-lysine-coated Petri dishes containing 1.5 mL pre-warmed culture medium. Medium exchange was performed in primary cortical cultures 4 days after seeding. Thereafter, medium together with non-adherent cells and cell debris were removed and a culture medium with KCl (final concentration of 25 mM) was added and the cells were cultivated in this medium without further exchange.

Plasmid Preparation

The plasmid pEGFP-N1, which codes for green fluorescent protein (GFP), was used in all experiments. Plasmid pEGFP-N1 was propagated in the competent Escherichia coli K-12 strain, and purified using Qiagen HiSpeed Plasmid Purification Maxi kits (Qiagen, Hilden, Germany). The pDNA concentrations were determined spectrophotometrically at 260 nm, and confirmed by gel electrophoresis.

Lipofection Procedure

Lipofectamine 2000 (Gibco by Life Technologies) was used as the lipid vehicle. Twenty-four hours before transfection, the growth medium was replaced with medium without antibiotics and antimycotics. The Lipofectamine 2000-pDNA complexes were prepared as follows: for each transfection sample, the required volume (2 – 4 µL) of Lipofectamine 2000 stock solution was diluted in 100 µL Opti-MEM without serum (Gibco by Life Technologies). After 5 minutes, the Lipofectamine 2000 solution was combined with the previously prepared DNA solution (5 µg pDNA diluted in 100 µL Opti-MEM, without serum). The solution was left to stand at room temperature for 20 minutes, to allow to form Lipofectamine 2000-pDNA complexes (lipoplexes).

Subsequently, the medium without antibiotics and antimycotics in each well of the cell cultures was changed to Opti-MEM (without serum) and again replaced for 6 hours with 200 µL lipoplexes. After lipofection, the medium lipofection complex solution was replaced with full Opti-MEM medium. The cells were then incubated for 24 hours in the cell culture incubator before further experiments. The cells in the control samples were not exposed to the lipofection.

Gene Electrotransfer Procedure

Before GET, the cell culture growth medium was replaced with 200 µL of the electroporation medium containing 25 µg/mL pDNA (5 µg pDNA per sample) for 3 minutes. Immediately after pulse delivery, 25% (v/v) FBS was added. The cells were then incubated for 5 minutes at 37°C, after which 1 mL of Opti-MEM with 10% FBS was added. The cells were then grown for 24 hours in the humidified 5% CO_2 atmosphere at 37°C. The cells in the experimental group received GET with ECM 830 electroporation system (BTX Instrument Division, Harvard Apparatus, MA) under the condition of 200 V at the pulse time of 25 ms which was optimized in our previous report to induce efficient transfection (18). The cells in the control samples were not exposed to the electric pulses.

Determination of Neuronal Viability and Transfection Efficiency

Cell viability (% viability) after the lipofection and GET treatments was determined as the ratio between the number of Hoechst 33342 (Molecular Probes, Grand Island, NY, USA) -stained nuclei counted in the treated sample to those in the control sample (19), expressed as a percentage of viability according to the equation: % Viability = 100 x N/N_{Cont}, where N is the number of all counted Hoechst-labeled cells in a given sample and N_{Cont} is the number of all counted Hoechst-labeled cells in a control sample. The number of viable cells was determined 24 hours after the lipofection or GET procedures were completed.

The efficiencies of the lipofection and GET were determined 24 hours after each procedure was completed. The transfection efficacy (% transfection) is defined as the ratio between the number of GFP-positive cells and the number of all viable neurons, expressed as the percentage of transfection according to the equation: % transfection = 100 x N_{GFP}/N_{total}, where N_{total} represents the number of all viable neurons, and N_{GFP} represents the number of all transfected neurons (GFP-positive) for each sample. The number of GFP-positive cells and the total number of viable cells were determined with a confocal laser microscope (FV-1000, Olympus, Tokyo, Japan), by counting 10 visual fields per culture sample (as 60 visual fields per independent experiment).

Sholl Analysis

The "Sholl analysis" of the imageJ analysis system (http://rsb.info.nih.gov/ij/download.html, NIH) (20) was used to evaluate the dendritic arborizations of primary cultured cortex neurons transfected with GFP-plasmids. First, the cells and processes of interest were outlined to exclude adjacent cells or areas of non-specific immunoreactivity. Templates of concentric circles increasing in radii by 10 µm were overlaid onto the center of a digitized cell soma (Fig. 2A). For each cell, densitometric thresholds were set to remove background labeling and identify detailed cellular processes. Single pixels of immunoreactive labeling above the threshold of detection were automatically removed to reduce false positives. The total number of objects above the threshold intersecting each circle was counted. The total length of processes and the number of intersections (branching complexity) for all rings were generated for each neuron. The transfections were repeated for 6 batches per condition, the mean of 12 neurons from each batch was used to get the averaged measurements (n = 6).

Results

Neuronal Viability and Transfection Efficiency of Primary Cultured Cortex Neurons with Lipofection or GET

To establish the optimal conditions for neuronal lipofection, the present investigation determined and compared the lipofection efficiencies of different lipoplexes (5 μg DNA mixed with 2.0, 3.0, or 4.0 μL Lipofectamine 2000 stock solution to make a final volume of 200 μL lipoplexes, as described in methods) after 24 hours incubation. According to our previous studies, the optimized GET condition for the efficient transfection is 200 V/25 ms (18). Thus, we chose this condition and compared the neuronal viability and transfection efficiency with that of lipofection (Fig. 1). One way ANOVA revealed that there was significant difference among these treatments on the neuronal viability [$F(3,23) = 14.29$, $P < 0.0001$]. Bofferroni's multiple comparison test revealed that the significant difference existed between 2 and 4 μL lipofection groups ($t = 3.53$, $P < 0.05$), 2 μL

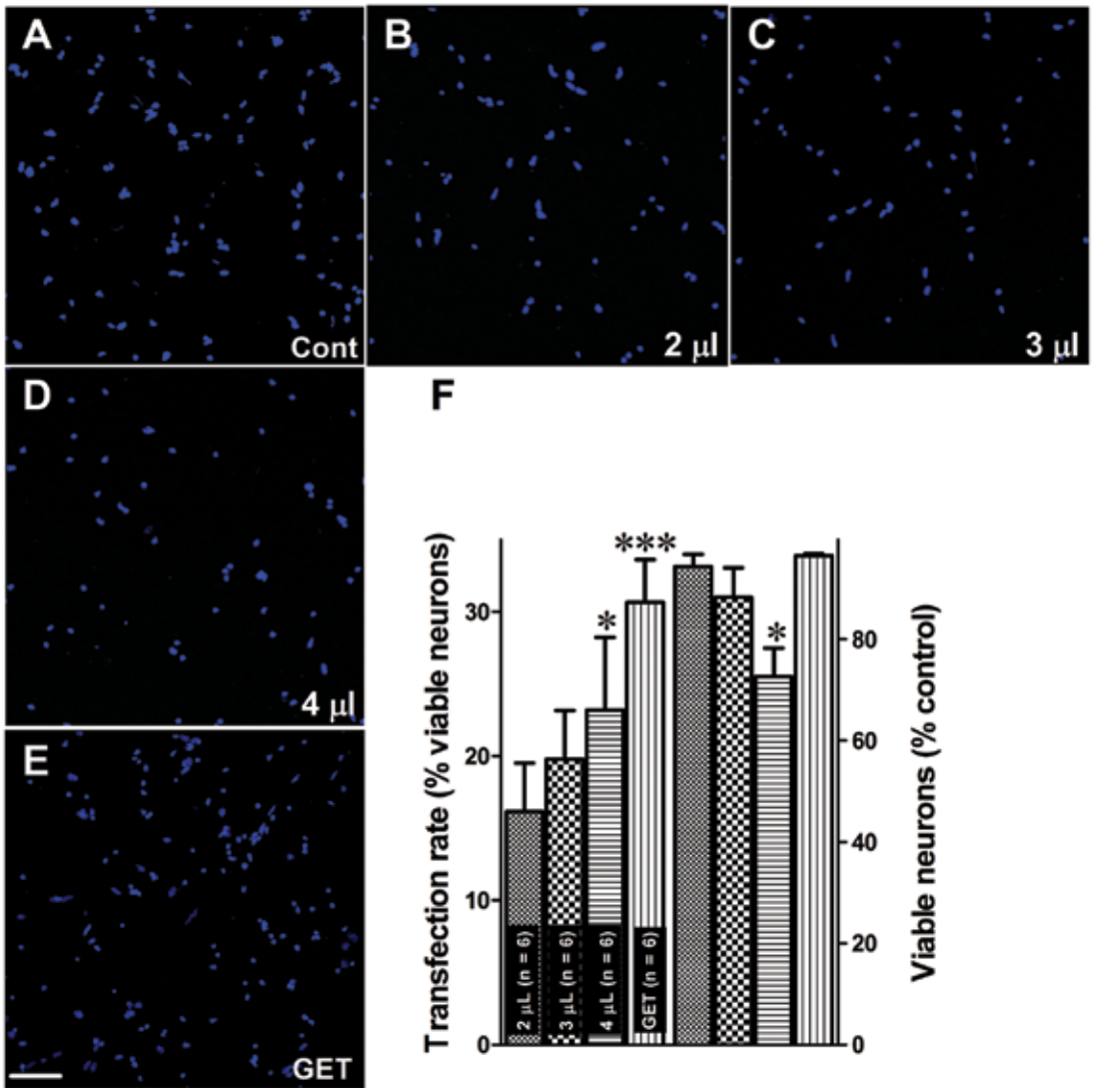

Fig. 1. *Neuronal viability and transfection efficiency of primary cultured cortex neurons after lipofection or GET. Represent Hoechst-33342 nuclei staining of primary cultured cortex neurons receiving no transfection (Cont, A), 2 (2 μL, B), 3 (3 μL, C), or 4 (4 μL, D) μL lipotection as well as GET (GET, E) treatment. Five μg pDNA was kept in all the experiments for lipofection or GET. Data were expressed as mean ± SD (N = 6 batches of culture). *, P < 0.05; ***, P < 0.001; statistically different from the primary cultured cortex neurons receiving 2 μL Lipofectamine trasfection. Scale bar in E = 100 μm.*

lipofection and GET group (t = 6.29, $P < 0.001$), as well as 3 μL lipofection and GET group (t = 4.46, $P < 0.001$). The viability of primary cultured neurons receiving 2, 3, 4 μL Lipofectamine transfection or GET was 94.28 ± 2.51%, 88.28 ± 5.76%, 72.63 ± 5.61%, or 96.45 ± 0.41%, respectively. Meanwhile, one way ANOVA revealed that there was significant difference among these treatments on the transfection efficiency [F(3,23) = 10.75, $P = 0.0002$]. Bofferroni's multiple comparison test revealed that the significant difference existed between 2 and 4 μL lipofection groups (t = 3.40, $P < 0.05$), 2 μL lipofection and GET group (t = 3.76, $P < 0.01$), as well as 3 μL lipofection and GET group (t = 5.40, $P < 0.001$). The transfection rate for primary cultured neurons receiving 2, 3, 4 μL Lipofectamine transfection or GET was 16.13 ± 3.38%, 19.78 ± 3.38%, 23.20 ± 5.02%, or 30.67 ± 2.94%, respectively.

All these data suggested that GET has less influence on neuronal viability after transfection, while neuronal viability decreased along with increased volume of Lipofectamine used for transfection. For gene therapy, another important issue identified is that transfected neurons should demonstrate similar dendritic arborization of their *in vivo* counterparts, so as to function normally. It is difficult to differentiate dendrites from axons in primary cultured neurons. The present investigation further investigated dendritic arborizations by looking at the total length of processes and the process intersections with circles using Sholl analysis (e.g., process was referred as dendrites in the following part).

The Influence of Lipofection and GET on the Dendritic Arborization of Primary Cultured Cortex Neurons

In Sholl analysis, 2 parameters are commonly used to evaluate the complexity of dendritic arborization, i.e., the intersections with circles of different radii from the soma and the total length of dendrites. Calculation of these 2 parameters from primary cultured neurons was performed receiving different transfection treatments (Fig. 2). The represented high magnification images from primary cultured neurons receiving 2 (Fig. 2A), 3 (Fig. 2B), or 4 (Fig. 2C) μL Lipofectamine transfec-

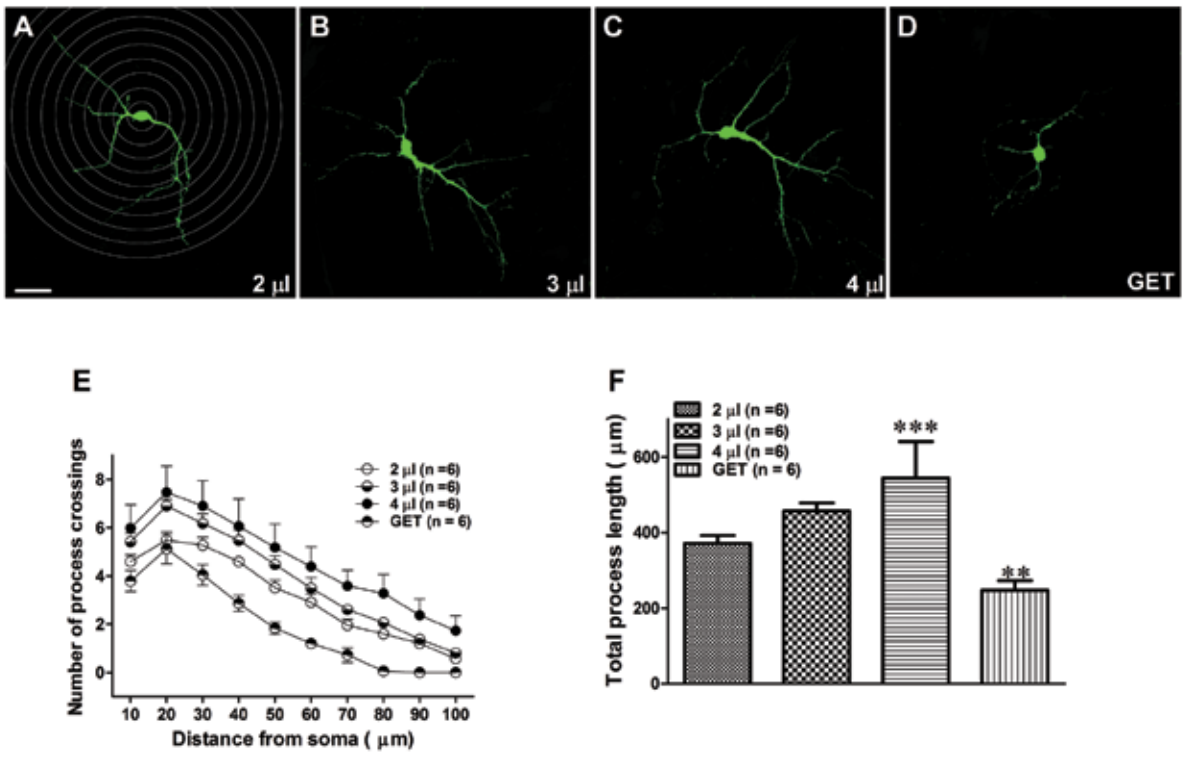

Fig. 2. *The dendritic arborizations of primary cultured cortex neurons with lipofection or GET. Represent GFP staining of primary cultured cortex neurons receiving 2 (2 μL, A), 3 (3 μL, B), or 4 (4 μL, C) μL lipotection as well as GET (D) treatment. The dendritic intersections with circles of different radii (E) and total process length (F) from 6 batches of primary cultured cortex neurons per condition were statistically analyzed. Data were expressed as mean ± SD (N = 6 batches of culture). **, P < 0.01; ***, P < 0.001; statistically different from the primary cultured cortex neurons receiving 2 μL Lipofectamine trasfection. Scale bar in A = 20 μm.*

tion as well as GET (Fig. 2D) demonstrated that lipfection is superior to GET related to inducing complex dendrite arborization. The statistical analysis from 6 repeated neuronal cultures per condition (12 neurons/batch *6 = 72 neurons) also revealed group difference in number of intersections with circles of different radii from the soma (Fig. 2E) and total length of dendrites (Fig. 2F). Two-way ANOVA revealed significant differences among the 4 groups, and the differences were derived from intersection distance [$F(9,180) = 1392$, $P < 0.001$], the treatment [$F(3,180) = 38.02$, $P < 0.001$], and the interaction between distance and treatment [$F(27,180) = 8.60$, $P < 0.001$], respectively. The contributions of these 3 factors to total variation were 71.93% (for distance), 21.87% (for treatment), and 1.33% (for interaction), respectively. These data suggest that first, the complexity of dendritic arborization is distance dependent since the most complex arborization exists at the trajectory of circles within a 20 – 50 μm radii; second, different transfection condition significantly affects the dendritic arborization, and therefore, may alter neuronal functions of these target neurons.

Furthermore, one way ANOVA revealed significant differences in the total process lengths among different groups [$F(3,23) = 35.46$, $P < 0.001$]. The significant differences mainly derived from the comparisons between 2 and 4 μL ($t = 5.74$, $P < 0.01$) Lipofectamine treatment groups as well as the significantly longer process of 2 ($t = 4.14$, $P < 0.01$), 3 ($t = 6.97$, $P < 0.01$), or 4 ($t = 9.88$, $P < 0.01$) μL Lipofectamine treatment groups when compared with GET. The total process length of primary cultured neurons receiving 2, 3, or 4 μL Lipofectamine treatment, as well as GET treatment, was 371.94 ± 20.55, 457.08 ± 21.28, 544.17 ± 96.18, or 247.64 ± 25.04 μm, respectively.

The Influence of Lipofection and GET on the Distance Dependent Process Arborization of Primary Cultured Cortex Neurons

Sholl analysis revealed that lipofection is superior to GET in inducing complex dendritic arborization. However, the dendrite segments at different distance from the soma plays different roles (21). The alterations at different dendritic segments contribute to varied changes in neuronal function. Sholl analysis could not answer the question whether lipofection and GET differently affect dendrites. Thus, our present investigation performed population analysis on the distance dependent distribution of intersections from all the 72 neurons per condition (Fig. 3). The cumulative curve showing the intersections at different radii (Fig. 3A) revealed similar findings from Fig. 2. It is difficult to reveal the varied influence of lipofection or GET on the distance dependent distribution of intersections. Thus, cumulative percentage was plotted of intersections on the different radii (Fig. 3B). Kruskal-Wallis H test revealed that there were no differences among these 4 data sets ($P = 0.54$), suggesting that the distance dependent feature of dendritic arborization had not been altered by the different treatments.

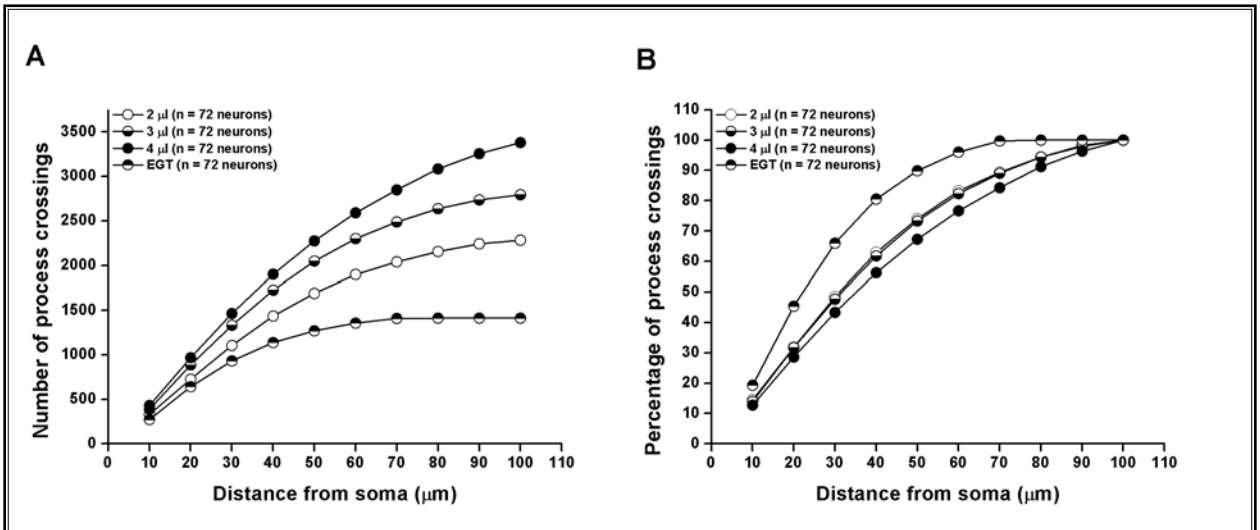

Fig. 3. *The influence of lipofection and GET on the distance dependent process arborization of primary cultured cortex neurons. The cumulative curves of absolute intersections (A) or percentage (B) from 72 neurons per-condition were constructed.*

Discussion

In the current study, our investigation revealed that GET introduced less cytotoxicity to primary cultured cortex neurons compared with lipofection. However, the primary cultured cortex neurons receiving GET demonstrated less complexity in dendritic arborizations. Although both lipofection and GET are non-viral transfection methods, to enable the following investigation on the dendritic arborization and neuronal function, lipofection showed superiority over GET. Whether longer culture time can compensate for the GET associated less complexity needs to be further investigated.

Advances in genetic research have offered a set of new therapeutic techniques that can be applied for in vitro and *in vivo* genetic manipulations, which might aid in development of new treatments for neurological deficit and pain-related disease states related to alterations in dendritic arborization (15). As gene therapy using viral transfection has raised concerns about immunogenicity, non-viral methods are being exploited to provide efficient and safe *in vivo* gene transfer. Lipofection and GET, as 2 main non-viral gene transfer methods, have been previously compared with regard to their transfection efficiency and toxicity in cultured human myoblasts (22). GET demonstrated comparable transfection efficacy with less cytotoxicity, and might be advantageous for *in vivo* application in skeletal muscle (22). However, their comparison in primary cultured cortex neurons has not been performed to date.

As a non-viral transfection method, lipofection is considered to be a relatively simple and less toxic method for transfection of genetic material, in comparison to viral transfection (23). Lipofection also has some advantages in comparison with other non-viral transfection methods (24), such as simplicity and low toxicity. In the present study, our investigation tested the effects of lipid vehicle concentration on lipofection efficiency using Lipofectamine 2000 in primary cultured cortex neurons, while the concentration of the pDNA was kept constant. The transfection efficiency achieved under our conditions was comparable with several other studies that have been performed with other neuronal cultures using Lipofectamine 2000 (25). However, the cell type specific cytotoxicity of Lipofectamine 2000 remains a serious issue for its practical application in a clinical setting. In the current study, the cytotoxicity of lipofection under the condition of 4 μL Lipofectamine accounted for significant neuronal death (27.37%). Thus, to reduce neuronal toxicity, other new-generation Lipofectamines need evaluation (26).

For gene therapy, GET has an advantage over lipofection in that no additional chemicals are used. GET is generally regarded as the method with the greatest potential in gene therapy, and it is therefore essential to gain better insights into the parameters that determine its efficacy. Previously, our laboratory investigated optimized parameters for efficient gene transfer in primary cultured cortex neurons and has determined that 200V/25 ms or 250V/15 ms are the most suitable conditions to reach a higher transfection rate; however, dendritic arborization was not compared with other gene transfer methods (18). The optimized GET condition enables an efficient transfection with less toxicity. However, dendritic arborization after GET remains an issue before it is recommend for actual *in vitro* or *in vivo* application.

We investigated the dendritic arborizations of primary cultured cortex neurons receiving different treatments to elucidate the potential influence of gene transfection. GET, although inducing an efficient transfection, did not lead to complex dendritic arborization. These features made GET unsuitable for neuronal gene transfer at present. On the contrary, along with the increase of Lipofectamine volume, the complexity of dendritic arborization increased in a dose-dependent manner, reaching its peak with a volume of Lipofectamine of 4 μL. Considering the neuronal cytotoxicity, a transfection rate of 19.78% with viability of 88.28% was obtained when the primary cultured cortex neurons received 3 μL Lipofectamine treatment highlighting many potential considerations for future *in vitro* lipotransfection of cortex neurons.

To make GET applicable for research or clinic purposes, there are several issues to be resolved. First, whether extended culture of GET neurons can increase complexity of dendritic arborization needs to be extensively investigated and better clarified. Second, the underlying mechanisms for lower complexity of dendritic arborization after GET should be studied such that additional strategies might be applied to GET to improve dendritic arborizations. Given the fact that pain states, stress mediated pathogenesis, and certain CNS diseases are linked to abnormalities associated with dendritic arborization, this emerging technique with additional refinement could play a role in mediating or modulating this complex process as a potential therapeutic modality in the future (18).

Conclusion

In summary, our study demonstrated that lipofec-

tion shows superiority over GET in efficiency of transfer genes to cultured neurons, while enabling complex dendritic arborization. These findings, offer a promising tool to investigate underlying mechanisms of CNS diseases, stress states, and pain states. However, future studies focusing on how to improve dendritic arborizations after GET are warranted in order to make this technique a promising gene delivery tool with therapeutic potential.

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