Cationic Amino Acid Based Lipids as Effective Nonviral Gene Delivery Vectors for Primary Cultured Neurons

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S *Supporting Information*

ABSTRACT: The delivery of specific genes into neurons offers a potent approach for treatment of diseases as well as for the study of neuronal cell biology. Here we investigated the capabilities of cationic amino acid based lipid assemblies to act as nonviral gene delivery vectors in primary cultured neurons. An arginine-based lipid, $Arg-C_3-Glu2C_{14}$, and a lysine-based lipid, $Lys-C_3-Glu2C_{14}$, with two different types of counterion, chloride ion (Cl^-) and trifluoroacetic acid (TFA^-), were shown to successfully mediate transfection of primary cultured neurons with plasmid DNA encoding green fluorescent protein. Among four types of lipids, we optimized their conditions such as the lipid-to-DNA ratio and the amount of pDNA and conducted a cytotoxicity assay at the same time. Overall, $Arg-C_3-Glu2C_{14}$ with TFA^- induced a rate of transfection in primary cultured neurons higher than that of $Lys-C_3-Glu2C_{14}$ using an optimal weight ratio of lipid-to-plasmid DNA of 1. Moreover, it was suggested that $Arg-C_3-Glu2C_{14}$ with TFA^- showed the optimized value higher than that of Lipofectamine2000 in experimental conditions. Thus, $Arg-C_3-Glu2C_{14}$ with TFA^- is a promising candidate as a reliable transfection reagent for primary cultured neurons with a relatively low cytotoxicity.

Cationic amino acid based lipids (Non-viral gene delivery vectors)

Gene expression in primary cultured neurons

KEYWORDS: *Cationic amino acid based lipids, liposomes, gene delivery, transfection, primary cultured neurons, green fluorescent protein*
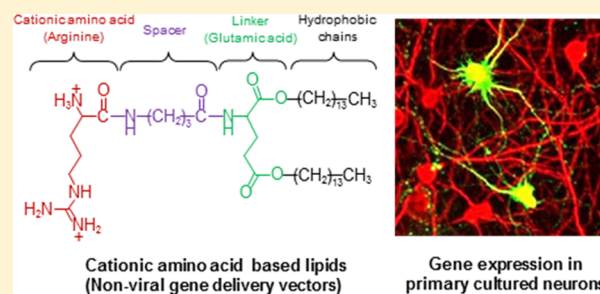
The central nervous system (CNS) is one of the most vital tissues of the body and abnormalities in its function underlie many debilitating and intractable neurological diseases, such as Alzheimer's disease and Parkinson's disease. Recently, it has been suggested that the delivery of plasmid DNA (pDNA) into CNS neurons to gain new gene expression patterns, or of small interfering RNA (siRNA) to knockdown specific genes might offer novel therapeutic strategies for many neurological diseases.[1-3] Transfection of pDNAs or siRNAs into primary cultured neurons also provides an important experimental technique for the study of neuronal cell biology, such as synapse formation and signal transduction; these investigations will provide greater insight into the mechanisms and functions of the brain at the molecular level.[4,5]

Transfection of nucleic acids into neurons is challenging, and various methods have been tried: viral vectors, electroporation, calcium phosphate, cationic polymers, and cationic liposomes.[6-8] Of these methods, the use of viral vectors is preeminent not only in research but also in clinical gene therapies. However, viral-based transfection requires P2-level containment for most of the viruses currently in use in the laboratory. Furthermore, there are other problems associated with the use of viral vectors, such as inducing inflammation and difficulties in their production in mass quantities.[9] For these reasons, alternative nonviral transfection methods have been explored and developed. However, the delivery of exogenous nucleic acids into CNS neurons with nonviral vectors is a challenge because of the high resistance of neurons to transfection and their high sensitivity to the materials used in such gene delivery vectors.[3,10]
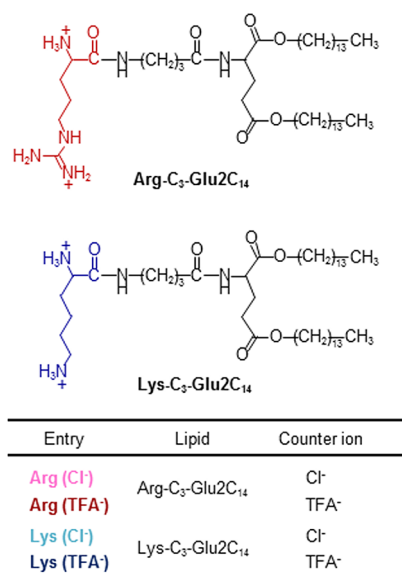
With regard to nonviral vectors, cationic lipids have the advantages of low cytotoxicity, ease of handling, and relatively higher transfection efficiencies than other nonviral gene delivery vectors.[11-14] Although cationic lipids are relatively toxic compared to neutral and anionic lipids, the cationic lipid assemblies have an essential advantage in that they spontaneously form lipoplexes with negatively charged nucleic acids such as pDNAs and siRNAs. Furthermore, it is believed that the resulting complexes electrostatically interact with the negatively charged cell membrane to mediate fusion and/or endocytosis, resulting in the delivery of nucleic acids into the cells.[15,16] Intravenous administration for nucleic acid delivery to central neurons is still challenging because of rapid systemic clearance of lipoplexes. Therefore, in vivo applications of cationic lipid carriers of nucleic acids including amino acid based lipids for neuronal delivery might be carried out with direct administration to the CNS including intraparenchymal injection, intraventricular injection, and intrathecal injection. Importantly,

transfection efficiency depends on controllable factors such as the type of cationic lipids and the mixing ratio of the cationic lipids and nucleic acids. Additionally, the efficiency is often influenced by the combination of the cationic lipids and cell type. To evaluate each of these factors, various types of cationic lipids have been synthesized and investigated for their efficiencies as transfection reagents.[17,18] Of the conventional transfection reagents based on cationic lipids, LA2000 (LA2000) is considered as the "gold standard" of the commercially available compounds, as it possesses high transfection efficiency across a range of cell types including neurons.[13] However, the transfection efficiency in neurons is still low compared to that of viral-based transfection methods, indicating that cationic lipids have room for further improvement in their transfection efficiency through optimization of the cationic lipid structure. In addition, the cationic lipids should possess low toxicity characteristics to neurons as well as to other cells.

In our previous studies, cationic amino acid based lipids were shown to have relatively high transfection efficiency and low cytotoxicity in various cell lines[19−21] and also in cell lines derived from neurons.[22,23] When compared with Lipofectamine2000, these findings suggested that cationic amino acid based lipids might be effective for use in transfection of primary cultured neurons in which transfection is often difficult. In the present study, we sought to identify the conditions for optimal transfection efficiency in primary cultured neurons using cationic amino acid based lipids.

The cationic amino acid based lipids Arg-C_3-Glu2C_{14} and Lys-C_3-Glu2C_{14} (Figure 1) were synthesized, and assemblies of

| Entry	Lipid	Counter ion
Arg (Cl⁻)	Arg-C_3-Glu2C_{14}	Cl⁻
Arg (TFA⁻)		TFA⁻
Lys (Cl⁻)	Lys-C_3-Glu2C_{14}	Cl⁻
Lys (TFA⁻)		TFA⁻

Figure 1. Chemical structures of the cationic amino acid based lipids Arg-C_3-Glu2C_{14} and Lys-C_3-Glu2C_{14}. These lipids were further characterized by their counterion as Arg (Cl⁻), Arg (TFA⁻), Lys (Cl⁻), and Lys (TFA⁻).

these lipids were prepared as described in our previous reports.[21,23] Many conventional cationic lipids used for transfection are unable to form stable self-assemblies. Therefore, helper lipids such as dioleoyl phosphatidylethanolamine (DOPE) are added to the cationic lipid mixture to reinforce the formation of stable liposomes.[17,24] The liposomes are then introduced into cells via endocytosis. It is believed that DOPE

functions to mediate endosomal escape by a pH-triggered phase transition. By contrast to the conventional cationic lipids, the amino acid based lipids used here can form stable cationic liposomes by themselves and the liposomes mediate efficient transfection of cultured cells.[23] Therefore, it is expected that the amino acid based lipids would possess a function of both of cationic lipids and helper lipids. This self-assembly property of cationic amino acid based lipids facilitates their handling and makes them convenient for use as transfection vectors.
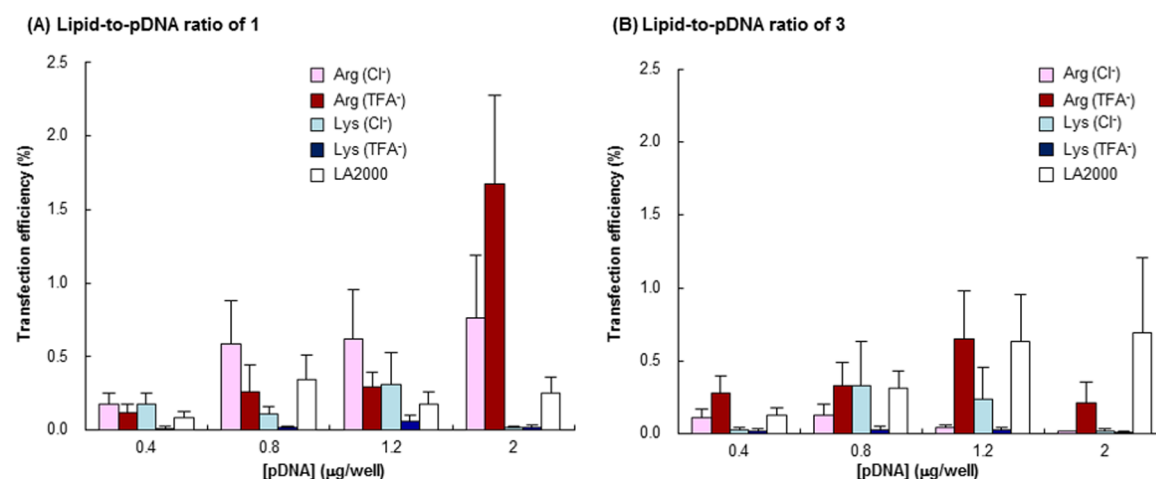
Transfection efficiencies of the cationic amino acid based lipid assemblies were evaluated in primary cultured neurons. The cells were obtained from mouse fetal hippocampi and cultured for 17−20 days in vitro before transfection. The synaptic fine structure of neurons matures in primary culture for 19−22 days.[25,26] Furthermore, synaptic plasticity in cultured hippocampal neurons is expressed only in cells >18 days in vitro.[27] However, it is widely experienced that gene transfection into old primary neuron culture (e.g., >14 days in vitro) results in very poor transfection efficiency with lower viability of neurons. Therefore, we targeted 17−20 days in vitro as the transfection time. Cytotoxicity of the cationic lipids was evaluated by immunocytochemistry in addition to morphological features. Ratio of neurons to total number of cells in the primary culture was estimated to 15.5 ± 1.4% by staining with an anti-MAP-2 antibody and 4′,6-diamidino-2-phenylindole (DAPI) (Supporting Information Figure S1). We used pDNA encoding a green fluorescent protein gene (GFP) (pEGFP-N1) for transfection experiments; GFP is often used as a reporter in neurobiological research due to its minimal toxicity to neurons. In addition, the distribution pattern of expressed GFP proteins can be monitored by fluorescence microscopy together with the morphology of the transfected cells. Neurons have highly polarized structures with different membrane compositions in different compartments such as the soma and the axon. After transfection with cationic amino acid based lipids, GFP was observed not only in the cell bodies but also in dendrites and axons (Figure 2B). In the field of view illustrated in Figure 2, nine neuronal somata with their processes were immunostained using the anti-MAP-2 antibody; three of the somata expressed GFP. It has been reported that lipofection can have adverse effects on neuronal morphology depending on the cell type and transfection reagents.[28] In general, transfection with cationic liposomes is performed by applying the lipoplexes (liposome−pDNA complexes) to cells for a few hours in the absence of serum; the cells are then washed, the culture medium is replaced with fresh one containing serum or equivalent, and the cells are incubated for a few more days. We tried this method for the primary cultured neurons; however, this approach appeared to result in cell damage (data not shown). Previously, we confirmed that lipoplexes could transfer a gene even in the presence of serum, although transfection efficiency decreased. Thus, we transfected primary cultured neurons in the presence of B27 supplement and exposed the cells to the lipid−DNA complexes for 2 days without a wash step. The number of GFP-expressing neurons increased by this method. This result indicates the high sensitivity of primary cultured neurons and suggests that high transfection efficiency can be achieved by applying lipid−DNA complexes to primary cultured neurons over time in the presence of B27 supplement. With this transfection protocol, no obvious morphological differences were observed before and after transfection or between cells expressing and not expressing GFP.
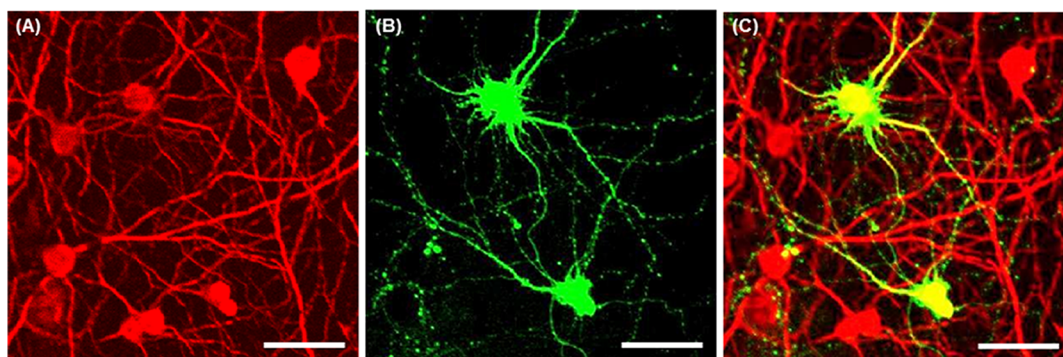
Figure 2. pEGFP-N1 transfected neurons viewed using a confocal microscope. (A) Neurons stained using an anti-MAP-2 antibody. (B) EGFP expression in some of the neurons. Arg (Cl$^-$); lipid-to-pDNA ratio of 1, [pDNA] = 0.8 μg/well. (C) Merged image of (A) and (B). Scale bar = 50 μm.
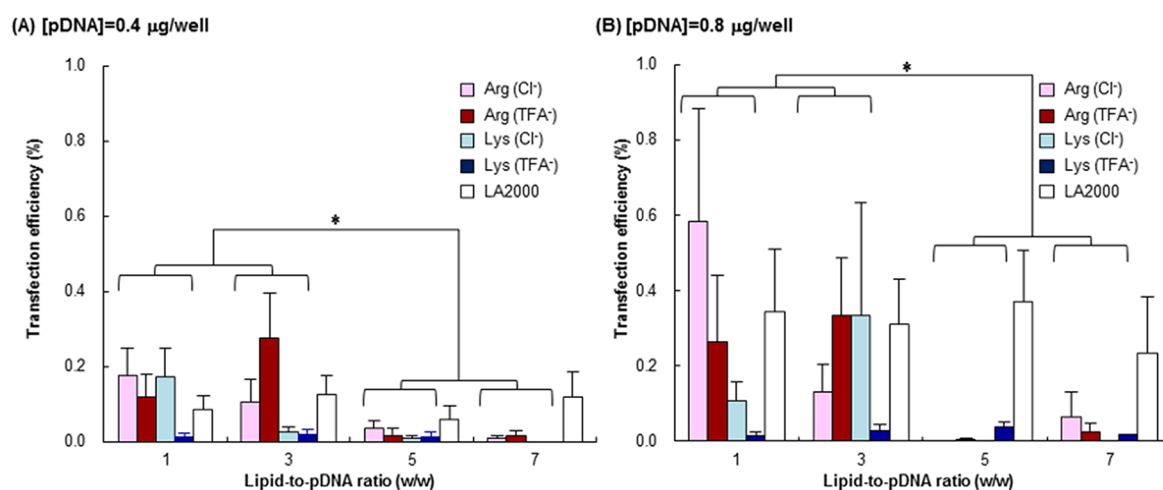
Figure 3. Effect of varying a lipid-to-pDNA ratio on the transfection efficiency of cationic amino acid based lipid assemblies compared to LA2000. The total amounts of pDNA per well are (A) 0.4 μg and (B) 0.8 μg. The concentrations of lipid are (A) 2, 6, 10, and 14 μg/mL at the lipid-to-pDNA ratios of 1, 3, 5, and 7, and (B) 4, 12, 20, and 28 μg/mL at the lipid-to-pDNA ratios of 1, 3, 5, and 7, respectively. Data are shown as means $\pm$ SEM of three or more replicate experiments. Statistical significance was determined by a two-way analysis of variance (ANOVA) (*$P < 0.05$).

Figure 4. Effect of total amount of pDNA on the transfection efficiency of cationic amino acid based lipid assemblies compared to LA2000. Lipid-to-pDNA ratios of 1 (A) and 3 (B) were used. The concentrations of lipid are (A) 2, 4, 6, and 10 μg/mL at [pDNA] of 0.4, 0.8, 1.2, and 2 μg/mL, and (B) 6, 12, 18, and 30 μg/mL at [pDNA] of 0.4, 0.8, 1.2, and 2 μg/mL, respectively. Data are shown as means $\pm$ SEM of eight replicate experiments. Significant dose-dependent increase of the Arg (TFA$^-$) value at a lipid-to-pDNA ratios of 1 were observed in a Kruskal–Wallis test ($P < 0.05$).

Transfection was performed under various conditions to optimize parameters such as the lipid-to-pDNA ratio and the quantity of the lipid–DNA complex. The ratio of GFP-expressing cells was used as an index of transfection efficiency. Since the structures of both the cationic lipid and counterion have a critical influence on transfection,[21,29] we tested the lipids

with two different counterions: chloride ion (Cl⁻) and trifluoroacetic acid (TFA⁻). As expected, the identity of the counterion influenced transfection efficiencies in primary cultured neurons (Figure 3). However, the effect of the counterion varied with the lipid-to-pDNA ratio and the identity of the cationic lipids. It was clear that the lipid-to-pDNA ratio was a critical factor for transfection efficiency regardless of the identities of the cationic amino acid based lipids or the counterions. Regarding lipid carriers excluding LA2000, with [pDNA] = 0.4 and 0.8 μg/well, the lipid-to-DNA ratios of 1 and 3 resulted in significantly higher transfection efficiency than the ratios of 5 and 7 ($P < 0.05$) (Figure 3) without significant difference among the carriers except for the extremely low transfection efficiency of Lys (TFA⁻). And the 0.8 μg/well pDNA dose yielded higher transfection efficiency than the 0.4 μg/well dose.

Therefore, we fixed the lipid-to-pDNA ratio to 1 and 3 and optimized the amount of pDNA (Figure 4). In case of the lipid-to-pDNA ratio of 1 at [pDNA] = 2 μg/well, Arg (TFA⁻) resulted in the highest transfection efficiency among the reagents ($P < 0.05$), whereas Arg (Cl⁻) showed still comparable efficiency but Lys (Cl⁻) and Lys (TFA⁻) showed negligible efficiency. In case of lipid-to-DNA ratio of 3 at [pDNA] = 1.2 μg/well, Arg (TFA⁻) tended to show the largest transfection efficiency, whereas Lys (Cl⁻) showed still comparable and Lys (TFA⁻) and Arg (Cl⁻) showed very small efficiency. The highest transfection efficiency of Arg (TFA⁻) at the lipid-to-pDNA ratio of 1, pDNA = 2.0 μg/well, can be considered to be higher than that of LA2000 in the same condition and then the highest transfection efficiency of LA2000 at a lipid-to-pDNA ratio of 3 and pDNA = 2.0 μg/well, although there was no statistical significance due to the rather large fluctuation of the results among trials (see Supporting Information Figure S3 for all experimental data). The percentage of non-neuronal cells expressing GFP was lower than 0.022% of the total non-neuronal cells, which was negligibly small compared with the transfection efficiency for neurons in these conditions. Thus we consider that the present transfection conditions are optimized only for primary cultured neurons.

Arg (TFA⁻) which showed the highest transfection efficiency among the cationic lipid carriers was used for a cytotoxicity assay and compared with LA2000. The lipid concentration in this cytotoxicity assay was set from 0 to 40 μg/mL, of which the range overlaps but differs from that used in the transfection assay (2−30 μg/mL). Although it has been speculated that the toxicity of cationic lipid−pDNA complexes (lipoplexes) is lower than that of cationic lipids without DNA owing to the neutralization of cationic charge of the cationic lipids by the negative charge of pDNA,[30] the presented cytotoxicity data of cationic lipid alone in Figure 5 is still of importance for evaluation of the performance of the cationic lipids and for estimation of the cytotoxicity of their lipoplexes. As shown in Figure 5, the number of live neurons decreased in a concentration-dependent manner with either Arg (TFA⁻) or LA2000 at more than 5 μg/mL. LA2000 showed higher cytotoxicity than Arg (TFA⁻) ($P < 0.01$, two-way ANOVA). With 10 μg/mL of Arg (TFA⁻), the survival rate of neurons was 71.1 ± 5.6%. The condition (2.0 μg pDNA/well and lipid-to-pDNA ratio of 1) was the optimal in transfection efficiency at this Arg (TFA⁻) concentration. On the other hand, LA2000 showed the highest transfection efficiency at the condition of 2.0 μg pDNA/well and lipid-to-pDNA ratio of 3, in which condition the concentration of LA2000 was 30 μg/mL. Taking
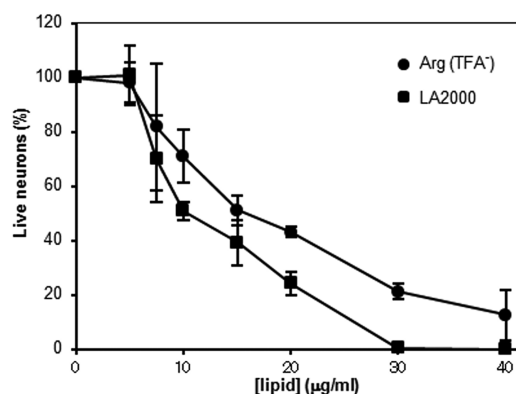
Figure 5. Cytotoxicity of cationic amino acid based lipid assemblies, Arg (TFA⁻), and LA2000 to primary cultured neurons as a function of lipid concentration. After 48 h of exposure with lipids, live neurons were stained and counted (Supporting Information Figure S2). Data are shown as means ± SD ($n = 3$). Number of surviving neurons is significantly different between Arg (TFA⁻) and LA2000 (two-way ANOVA, $P < 0.01$).

the fact that LA2000 contains helper lipids in addition to a cationic component into account, a much larger amount of LA2000 than Arg (TFA⁻) might be required for the highest transfection efficiency. However, the cytotoxicity assay indicated that 30 μg/mL of LA2000 alone damaged most of the primary cultured neurons. It was also shown that Arg (TFA⁻) less damaged neurons than LA2000 at any concentrations above 5 μg/mL. This lower cytotoxicity of Arg (TFA⁻) would be particularly important in effective transfection to delicate neurons.

In cell line cultures, cationic amino acid based lipids were found to have high transfection efficiencies when the lipid-to-pDNA ratio was in the range 10−20.[21,23] As the ratio increases, the net positive charge on the lipid−DNA complexes also increases resulting in more active electrostatic interactions of the lipid−DNA complexes with the negatively charged cell membrane. As described above, we found here that lower ratios gave better transfection efficiencies in the primary cultured neurons. Thus, the ratio of lipid to pDNA for optimal transfection efficiency differs significantly between primary cultured neurons and cell lines. In particular, it should be noted that the optimal conditions for cell lines failed to function in primary neuron cultures. This difference is a consequence of the sensitivity of primary neurons to the transfection procedure. In cell line cultures such as SH-SY5Y and PC-12, the optimal lipid concentrations for transfection are in the range of 20−30 μg/mL,[23] whereas primary neurons cannot survive in this lipid concentration range. Thus, optimal lipid concentration for transfection was found to be 10 μg/mL for primary cultured neurons.

In conclusion, we investigated cationic amino acid based lipids for their ability to transfect primary cultured neurons and determined the conditions that enabled efficient transfection and gene expression in mature primary neurons. Cationic amino acid based lipids offer valuable transfection reagents for primary cultured neurons with the optimized conditions described here.

■ METHODS

Animals. SIc:ICR mice were purchased from Sankyo Labo Service (Tokyo, Japan). Animal care was performed in accordance with institutional guidelines outlined in the Institutional Animal Care and

Use Committee of Waseda University. Throughout the experimental procedures, all efforts were made to minimize the number of animals used and their suffering.

Materials. Minimal essential medium (MEM), Hank's balanced salt solution (HBSS), penicillin and streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Nacalai Tesque (Kyoto, Japan). Neurobasal medium, B27 supplement, L-glutamine, trypsin, sodium pyruvate, and Lipofectamine2000 were purchased from Invitrogen (Carlsbad, CA). Polyethylenimine was purchased from Sigma-Aldrich (St. Louis, MO). DNase I was purchased from Roche (Basel, Switzerland). Anti-MAP-2 antibody was purchased from Santa Cruz (Santa Cruz, TX), and the secondary antibody, Alexa Fluor 594 conjugate anti-rabbit IgG, was purchased from Invitrogen. The synthetic lipids Arg-C_3-Glu2C$_{14}$ and Lys-C_3-Glu2C$_{14}$ (Figure S1, Supporting Information) and assemblies composed of these lipids were prepared as described in our previous articles.[21,23]

Plasmid DNA. A pEGFP-N1 vector (4.7 kb, CMV promoter; CLONTECH Laboratories, Inc.), which encodes enhanced green fluorescent protein (GFP), was amplified in ECOS Competent *E. coli* DH5α (NIPPON GENE) and purified using a QIAGEN Plasmid Mega Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions.

Preparation of Lipid–DNA Complex. The required amounts of the cationic assemblies, Arg (Cl$^-$), Arg (TFA$^-$), Lys (Cl$^-$), and Lys (TFA$^-$) (Figure 1), were transferred from stock solutions ([lipid] = 1 mg/mL) and diluted with neurobasal medium (GIBCO) without any supplement to a final volume of 10 μL. In the same way, the required amount of pDNA encoding GFP was transferred from a stock solution ([pDNA] = 1 mg/mL) and diluted with neurobasal medium (GIBCO) without any supplement to a final volume of 10 μL. Each of the cationic assemblies was mixed with the pDNA and agitated gently, followed by incubation at room temperature for 15 min. Each solution was diluted with neurobasal medium without any supplement to a final volume of 100 μL per well for transfection. Transfection was also performed using Lipofectamine 2000 (LA2000) as a control, according to the manufacturer's instruction.

Primary Cell Culture. Embryonic murine hippocampal neurons were obtained from pregnant SIc:ICR mice at 17 days gestation. The surface of each well of 48 well culture dishes was coated overnight at 37 °C with 200 μL of 0.04% polyethylenimine (PEI) and rinsed three times with sterile water. The coated plates were air-dried and stored at 4 °C for few days prior to use. Hippocampi from mouse embryos were incubated at 37 °C for 5 min in HBSS with 2% trypsin and 10% DNase I, rinsed three times in HBSS, and disaggregated in MEM medium supplemented with 1× B27, 1 mM sodium pyruvate, 500 U/mL penicillin, and 500 μg/mL streptomycin. The cells were seeded on PEI coated 48-well plates at a density of 4.0×10^4 cells/well (total volume per well of 200 μL). Cells were maintained at 37 °C and 5% CO$_2$. Every 3–4 days, half of the culture medium (100 μL) was replaced with neurobasal medium supplemented with 2 mM L-glutamine, 1× B27, 500 U/mL penicillin, and 500 μg/mL streptomycin. In order to determine the percentage of neurons in the primary cell culture, nuclei of all cells were stained with DAPI and the neurons were stained with an anti-MAP-2 antibody. The ratio of neurons was defined as a percentage of number of MAP-2 positive cells to that of all cells counted by DAPI stain.

Transfection Efficiency of Cationic Amino Acid Based Lipids. Transfection efficiencies of the cationic amino acid based lipids were assessed in the primary cultured neurons that had been cultured in vitro for 17–20 days. Half of the culture medium (100 μL) was replaced with neurobasal medium containing lipid–DNA complexes at different lipid-to-pDNA ratios and different total amounts of pDNA. After incubation at 37 °C and 5% CO$_2$ for 2 more days, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS; then the number of neurons expressing GFP per each well (0.75 cm^2) was counted under a fluorescence microscope, and the percentage of neurons expressing GFP out of the total number of neurons per each well was calculated as transfection efficiency (%).

Cytotoxicity Assay. The cytotoxicity of Arg-C_3-Glu2C$_{14}$ and LA2000 was evaluated in the primary cultured neurons that had been cultured in the same conditions with transfection experiments. The cell culture medium was replaced with a neurobasal medium containing various concentrations of Arg (TFA$^-$) or LA2000 (5–40 μg/mL). The cell cultures were incubated at 37 °C and 5% CO$_2$ for 48 h. The medium containing the cationic lipids was removed, and the cells were washed with PBS. Viability/cytotoxicity kit (Molecular Probes, Eugene, OR) assays were carried out following the manufacturer's protocol, in which cells were incubated in 1 μM calcein AM and 2 μM ethidium homodimer-1 at room temperature for 30 min. The cells were fixed with 4% paraformaldehyde in PBS and then stained with an anti-MAP-2 antibody and DAPI. The number of live neurons which were stained with calcein AM, anti-MAP-2 antibody, and DAPI was counted under a fluorescence microscope, and the ratio of number of live neurons at each lipid concentration to the number of live neurons in control wells which were not treated with lipids was calculated (Supporting Information Figure S2).

Statistical Analysis. Statistic calculations were performed using Microsoft Excel. Statistical significance was determined by a two-way analysis of variance (ANOVA), Kruscal-Wallis test, and one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Synthesis procedure. Representative fluorescence images of primary cultured cells stained with anti-MAP-2 antibody and 4′,6-diamidino-2-phenylindole (DAPI) for purity evaluation (Figure S1). Representative fluorescence images of primary cultured cells stained with calcein-AM and anti-MAP-2 antibody to count live neurons for cytotoxicity assay (Figure S2). All transfection efficiency data in the important trials (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Y.A. performed the cell culture, transfection experiments, and data analysis. R.H. and K.I. performed the cell culture, cytotoxicity assay, and data analysis. K.S. and H.N. provided advice on experiments and data analysis. S.R.S. synthesized and characterized the cationic amino acid based lipids. T.I. and S.T. conceived and supervised the project. All authors wrote the paper.

Notes

The authors declare no competing financial interest.

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