Treatment of neurological disorders by introducing mRNA in vivo using polyplex nanomicelles

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Abstract

Sensory nerve disorders are difficult to cure completely considering poor nerve regeneration capacity and difficulties in accurately targeting neural tissues. Administering mRNA is a promising approach for treating neurological disorders because mRNA can provide proteins and peptides in their native forms for mature non-dividing neural cells, without the need of entering their nuclei. However, direct mRNA administration into neural tissues in vivo has been challenging due to too unstable manner of mRNA and its strong immunogenicity. Thus, using a suitable carrier is essential for effective mRNA administration. For this purpose, we established a novel carrier based on the self-assembly of polyethylene glycol (PEG)-polyamino acid block copolymer, i.e. polyplex nanomicelles. To investigate the feasibility and efficacy of mRNA administration for the treatment of sensory nerve disorders, we used a mouse model of experimentally induced olfactory dysfunction. Intranasal administration of mRNA-loaded nanomicelles provided efficient, sustained protein expression for nearly two days in nasal tissues, particularly in the lamina propria which contains olfactory nerve fibers, with effectively regulating the immunogenicity of mRNA. Consequently, once-daily intranasal administration of brain-derived neurotrophic factor (BDNF)-expressing mRNA using polyplex nanomicelles remarkably enhanced the neurological recovery of olfactory function along with repairing the olfactory epithelium to a nearly normal architecture. To the best of our knowledge, this is the first study to show the therapeutic potential of introducing exogenous mRNA for the treatment of neurological disorders. These results indicate the feasibility and safety of using mRNA, and provide a novel strategy of mRNA-based therapy.

Keywords

1. Introduction

Sensory nerve disorders are difficult to cure completely considering poor nerve regeneration capacity and difficulties in accurately targeting neural tissues. Various bioactive factors, including nerve growth factor, brain-derived neurotrophic factor (BDNF) and neurotrophin-3, have been investigated for enhancing nerve regeneration and for nerve protection [1-4]. However, administering these factors in the form of recombinant proteins or peptides involves problems of poor stability under physiological conditions and a considerably short duration of action to achieve sufficient therapeutic effects; thus, their application for treatment has been hindered.

Administering mRNA is a promising approach for treating neurological disorders because mRNA has several advantages for providing therapeutic proteins or peptides to neural tissues; for example, mRNA can be used for protein or peptide expression in their native forms at a target site [5, 6]. The sustained synthesis of proteins resulting from the delivered mRNA can facilitate the synchronization between the kinetics of signal receptor expression and bioactive factor availability [7]. The combined use of two or more bioactive factors to provide better neurotrophic and neuroprotective effects is much easier than using recombinant proteins or peptides, because mRNA can be designed for the expression of any protein or peptide by simply altering the base sequence [8]. Indeed, this is also true for DNA administration, however, mRNA has a much higher potential for protein expression in neural tissues, because it can be used to produce proteins and peptides in mature non-dividing neural cells, without the need for entering their nuclei. Most importantly, the probability of mRNA randomly integrating into the genome is negligible, thus avoiding the aberrant expression of oncogenes as a result of insertional mutagenesis.

However, direct mRNA administration into neural tissues in vivo has been challenging due to two major issues: (1) introduced mRNA is often too unstable to achieve sufficient protein expression and (2) mRNA is strongly immunogenic and induces immune responses through its recognition by Toll-like receptors [6, 9, 10]. Thus, only a few studies have attempted in vivo mRNA administration[11-13], and even fewer have attempted targeting neural tissue.

Thus, using a suitable carrier is essential for effective mRNA administration. For this purpose, we recently established a novel carrier that was applicable for in vivo mRNA delivery based on the self-assembly of polyethylene glycol (PEG)-polyamino acid block copolymer, i.e. polyplex nanomicelles [14]. These nanomicelles have a core-shell structure surrounded by a PEG outer layer, with the inner core of a functionalized

polyamino acid, poly[N'-[N-(2-aminoethyl)-2-aminoehtyl] aspartamide] [PAsp(DET)] [15]. PAsp(DET) has a high capacity for enhanced endosomal escape due to pH-sensitive membrane destabilization as well as the unique characteristic of rapidly degrading into nontoxic forms under physiological conditions [16-18]. The nanomicelles [PEG-PAsp(DET) block copolymer / mRNA] were shown to be safe, stable mRNA carriers to allow in vivo mRNA introduction into the central nervous system [14] and other tissues and organs (unpublished data). In addition, they exhibited a pronounced effect for regulating immune responses that could be induced by exogenous mRNA [14]. This is likely due to the shielding effect of PEG to avoid mRNA recognition by Toll-like receptors on host immune cells.

To establish the feasibility and efficacy of mRNA administration for the treatment of sensory nerve disorders, we used a mouse model of experimentally induced olfactory dysfunction [19]. Neurogenesis occurs throughout life in the olfactory epithelium, where olfactory receptor neurons (ORNs) are continuously replaced by new ORNs [20]. During this process, neurotrophic factors such as BDNF play an important role in promoting the survival and differentiation of ORNs [21]. TrkB, a high affinity receptor for BDNF, is abundantly expressed by immature and mature ORNs and globose basal cells [22], suggesting that BDNF can likely be used as a therapeutic agent to treat pathological conditions involving ORNs.

In this study, we applied BDNF-expressing mRNA into the nasal cavity using polyplex nanomicelles. The nasal cavity is a potentially attractive target for administering therapeutic agents considering its relatively large surface area of nasal mucosa and good vascularity [23], although the abundance of RNase in nasal mucus is unfavorable for mRNA administration. However, the function of polyplex nanomicelles should be underscored for effectively introducing mRNA into neural tissues. To the best of our knowledge, this is the first study to show the therapeutic potential of introducing exogenous mRNA for the treatment of neurological disorders. The effects of BDNF-expressing mRNA on impaired olfactory sensation were investigated by comprehensive analyses, including behavioral assessments of mice and histopathological evaluations of the olfactory epithelium.

2. Materials and Methods

2.1 Preparation of mRNA

mRNA was prepared by in vitro transcription (IVT) of DNA templates that were constructed by inserting a protein-expressing fragment into a pSP73 vector (Promega,

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Madison, WI, USA) that included a T7 promoter. The protein-expressing fragments were obtained from pDNAs encoding photinus pyralis luciferase (pGL4; Promega), aequorea coerulescens GFP (AcGFP; Clontech, Mountain View, CA, USA) and BDNF (pUNO1-hBDNFa; InvivoGen, San Diego, CA, USA). pDNAs were used as a template for IVT after linearization by Nde-I. IVT was performed using an mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Invitrogen, Carlsbad, CA), followed by polyadenylation using a poly(A) tail kit (Ambion). To generate modified mRNA, modified ribonucleic acid triphosphates (5-methyl-CTP, pseudo-UTP and 2-thio-UTP; TriLink BioTechnologies, San Diego, CA) were added to the reaction solution at 20% of 5-methyl-CTP of total CTP, and 10% pseudo-UTP and 10% 2-thio-UTP of total UTP, following the procedures reported previously [11]. Prior to the administration to mice, the transcribed mRNA was purified using a QIAquick PCR purification kit (Qiagen) and analyzed for size and purity with an Agilent RNA 6000 Nano Assay on a BioAnalyzer 2100 (Agilent Technologies).

2.2 Animals

Balb/c mice (7–10 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan) and maintained under specific pathogen-free conditions. Mice were kept in individually ventilated cages, on a 12 h/12 h light/dark cycle, and provided food and water ad libitum. They were intraperitoneally injected with methimazole (Sigma-Aldrich Japan, Tokyo, Japan) at 150 mg/kg body weight to cause damage to the olfactory epithelium [19]. All experiments conformed to the guidelines of the University Committee for the Use and Care of Animals, University of Tokyo, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3 Preparation of polyplex nanomicelle solutions

PEG-PAsp(DET) block copolymers were synthesized as previously described [15]. PEG used in this study had a molecular weight of 12,000. The polymerization degree of the PAsp(DET) portion was determined to be 57 by ^{1}H-NMR analysis. To prepare polyplex nanomicelles, PEG-PAsp(DET) polymers and mRNA were separately dissolved in 10 mM HEPES buffer. At this stage, the concentration of mRNA was set to 300 μg/ml and that of PEG-PAsp(DET) was adjusted to obtain a ratio of amino groups in polymers to phosphate in mRNA (N/P ratio) of 3.

2.4 Intranasal administration

Mice were briefly anaesthetized with 2.5% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan), placed in a supine position, and instilled intranasally with 50 μl of a

solution containing 10 μg mRNA using a P200 Gilson pipetman.

To note is that we used mRNA solution comprised of 10 mM HEPES buffer for intranasal administration, because the hypotonic buffer would be available for intranasal administration without causing permanent damages on the nasal membrane [24-27]. It was reported that the hypotonic formulation would exert effects to temporarily open epithelial junctions and facilitate the uptake of drugs and genes [25].

2.5 Luciferase protein expression by bioluminescence assay

d-Luciferin substrate was dissolved in PBS, and the final volume was adjusted to obtain a concentration of 15 mg/ml. Bioluminescence emissions were measured using an IVIS imaging system (Xenogen, Alameda, CA, USA) 10 min after intraperitoneally injecting 200 μl of the d-luciferin solution (3 mg/mouse). Bioluminescence signals in the nose region were analyzed by background subtraction using Living Image Software version 2.50 (Xenogen).

2.6 Real-time RT-PCR for TNF-α, IL-6, and OMP mRNA in nasal tissues

Mice were sacrificed under deep anesthesia and total RNA was extracted from the nasal mucosa using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). mRNA expression for inflammatory cytokines (TNF-α and IL-6) and olfactory marker protein (OMP) was evaluated by real-time quantitative PCR (ABI Prism 7500 Sequence Detector; Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Assays (Mm00443258 for TNF-α, Mm00446190_m1 for IL-6, Mm00436450_m1 for OMP and Mm00607939 for β-actin). The relative mRNA expression for target genes was normalized using β-actin mRNA expression.

2.7 Histological examination after administering GFP-expressing mRNA into the nose

Mice were sacrificed and decapitated 24 h after administering GFP-expressing mRNA. The mandibles were discarded, and the trimmed heads were skinned. Serial sections (thickness, 5 μm) at the level of the anterior end of the olfactory bulb were prepared. These sections were stained with hematoxylin and eosin (H&E).

For immunohistochemical staining, 5-μm-thick frozen sections were prepared using an adhesive film-based method [28]. These sections were incubated at room temperature (RT) for 1 h with a blocking solution (PBS containing 2% fetal bovine serum and 0.1%Tween 20) to reduce non-specific antibody binding, and then incubated

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overnight with a rabbit anti-GFP monoclonal antibody (1:500 in the blocking solution; Invitrogen, Carlsbad, CA, USA). After several washes in PBS, the sections were incubated for 2 h at RT with an Alexa488-conjugated secondary antibody (1:200 in the blocking solution; Invitrogen, Carlsbad, CA, USA). After more washes with PBS, the nuclei were stained with Hoechst 33342 (1:300 in the blocking solution; Dojindo, Kumamoto, Japan). The sections were then observed under an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany) using a 20× EC Plan Neofuar objective (Carl Zeiss). As a negative control, the primary antibody was omitted from the reaction. There was no obvious labelling corresponding to immunostaining by the primary antibody (data not shown).

2.8 Olfactory function assessments

Buried food tests were performed as previously described, with some modifications [29]. Pieces of cheese were placed in animals' cages for at least 3 consecutive days before the test to familiarize the mice to the smell of the cheese. Mice were then individually placed in cages and deprived of food for 24 h. A small piece of cheese was buried 4 cm beneath the bedding surface in a randomly chosen corner of the cage. The time required to uncover the food, to grab or to eat it was recorded. These tests were performed 4 times at intervals of 10 min. The upper time limit to uncover the food was set at 5 min (300 s). If a mouse could not uncover the food within this time limit, the cut-off of 300 s was recorded.

2.9 Histopathological examination after inducing olfactory dysfunction and treatment with BDNF-expressing mRNA

Histopathological examinations were made by H&E staining and detecting OMP expression in the olfactory epithelium. For tissue preparation, mouse heads were fixed by immersion in 10% neutral buffered formalin (Muto Kagaku, Tokyo, Japan) at RT for 1 week, followed by decalcification in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.0) at 37°C for two weeks. After decalcification, specimens were washed, dehydrated in a graded ethanol series and embedded in paraffin. Serial sections (thickness, 5 μm) at the level of the anterior end of the olfactory bulb were prepared. These sections were then stained with H&E. For OMP immunostaining, rehydrated sections were immersed in 10 mM citrate buffer (pH 6.0; Dako Cytomation Japan, Kyoto, Japan) and autoclaved at 121°C for 20 min for antigen retrieval. Next, the sections were incubated for 1 h with a blocking solution [PBS, pH 7.4, containing 4% fetal bovine serum (Invitrogen, Tokyo, Japan), 0.1% Triton- X 100 and 0.1% sodium azide] at RT to reduce non-specific antibody binding, and then incubated with a goat anti-OMP antibody (1:5,000 in the blocking

solution; Wako Chemical USA, Richmond, VA) for 1 h at RT. After several washes with PBS, the sections were incubated for 1 h at RT with an Alexa546-conjugated secondary antibody (1:400 in the blocking solution; Invitrogen, Carlsbad, CA, USA). After more washes with PBS, the sections were mounted and the nuclei were stained with ProLong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad, CA, USA).

2.10 Statistical Analysis

Statistical analyses were performed using StatMate (Atoms, Tokyo, Japan). One-sample Kolmogorov–Smirnov tests were used to compare the data distributions against a normal distribution. Results are expressed as mean ± standard error of the mean (SEM). A two-tailed unpaired t-test or Mann-Whitney U-test was used for statistical comparisons. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1 Sustained protein expression in vivo after intranasal mRNA administration using polyplex nanomicelle

After incorporation into polyplex nanomicelles, luciferase-expressing mRNA (10 µg) was intranasally administered to BALB/c mice. We then evaluated luciferase expression using an IVIS imaging system; bioluminescence could be detected in nasal tissues 4 h after the administration (Fig. 1A), lasting for up to 48 h (Fig. 1B). In contrast, almost no luciferase expression was observed after administering an equal quantity of naked mRNA (Fig. 1A,B).
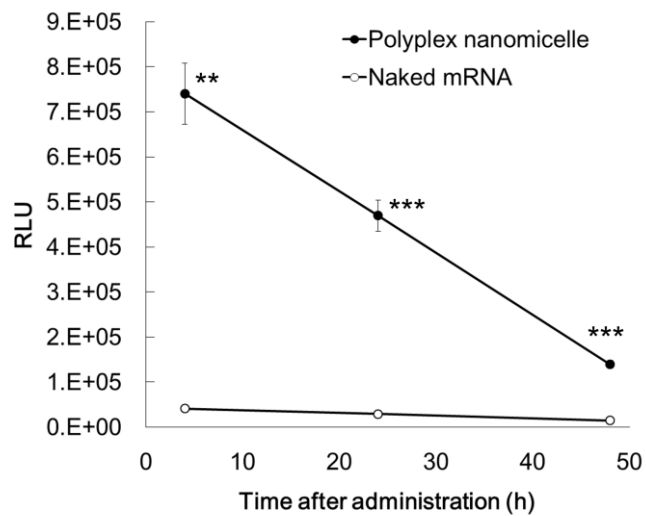
(A)
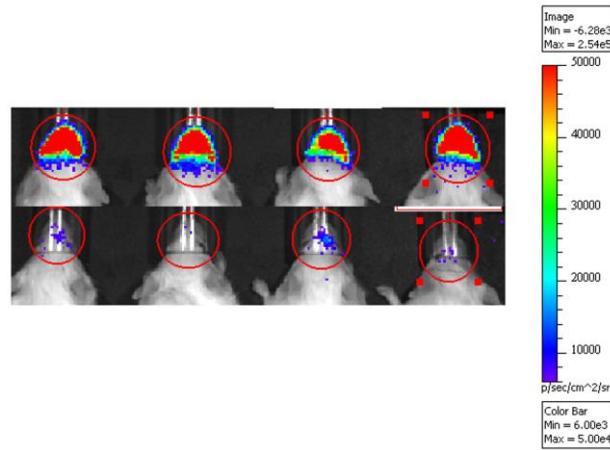
(B)

Figure 1

Bioluminescence after intranasally administering luciferase-expressing mRNA.

(A) Bioluminescence images obtained by an IVIS Imaging System 4 h after administering luciferase-expressing mRNA-loaded polyplex nanomicelles (upper) and an equal quantity of naked mRNA (lower).

(B) Time course of bioluminescence after intranasally administering mRNA using polyplex nanomicelles (closed circle) and naked mRNA (open circle). Statistical analyses were performed by two-tailed Student's t-test, *** p < 0.001, ** p < 0.01. RLU; relative luminescence units. Results are means ± SEMs (n = 4).

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Histological analysis was performed using GFP-expressing mRNA. GFP-positive staining was widely observed in the lamina propria, which contains olfactory nerve fibers and endings, and in the airway epithelial cells and the basal part of the olfactory epithelial cells (Fig. 2). However, no GFP-staining was detected in nasal septal cartilages and bones.
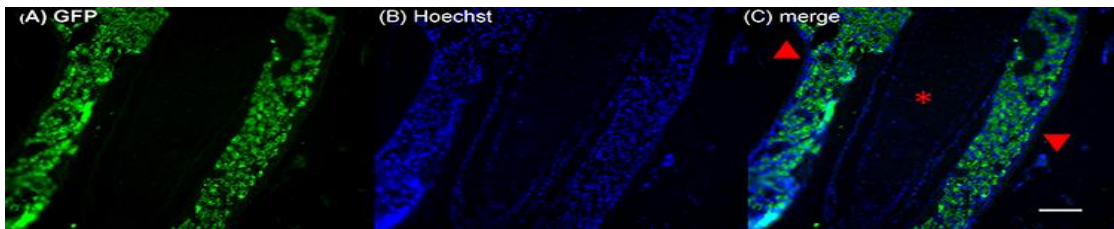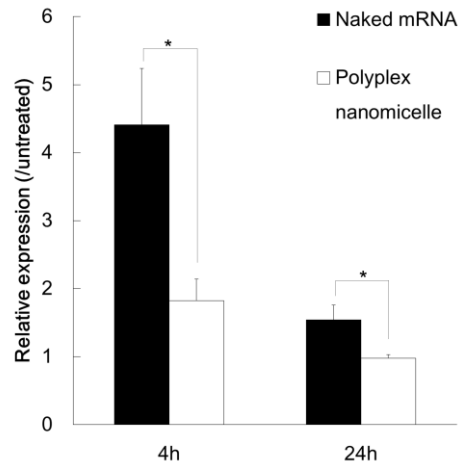

Figure 2

Histological analysis after intranasally administering GFP-expressing mRNA.

Mice were sacrificed and decapitated 24 h after administering GFP-expressing mRNA. (A) GFP expression visualized by immunostaining using an anti-GFP monoclonal antibody. (B) Cell nuclei stained by Hoechst. (C) Merged image. GFP-positive staining was widely observed in the lamina propria (arrowheads), but not in nasal septal cartilages and bones (asterisk). Scale bar: 50 μm.

The induction of proinflammatory cytokine genes after administration of luciferase-expressing mRNA was evaluated using real-time quantitative PCR (qPCR) by measuring the mRNA expression levels of genes encoding tumor necrosis factor (TNF)-α and interleukin (IL)-6 in nasal tissues (Fig. 3). At 4 h after mRNA administration using the nanomicelles, cytokine gene mRNA expression levels were increased compared with untreated control levels. However, at 24 h after administration, the expression levels had decreased to untreated control levels. In contrast, when mRNA was administered in the form of naked mRNA, the increase in cytokine gene induction was significantly higher than that using the nanomicelles, even at 24 h after the administration.
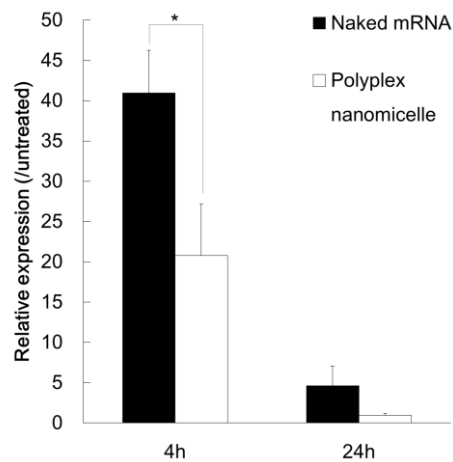
(A)

(B)

Figure 3

Induction of proinflammatory cytokine genes after intranasal mRNA administration.

Proinflammatory gene mRNA expression levels were evaluated 4 and 24 h after mRNA administration using real-time quantitative PCR (qPCR) by measuring the mRNA expression levels of genes encoding tumor necrosis factor (TNF)-α (A) and interleukin (IL)-6 (B) in nasal tissues administration of mRNA into the nasal tissue. mRNA was administered using polyplex nanomicelle (open bar) or in the form of naked mRNA (closed bar). Statistical analyses were performed by two-tailed Student's t-test, *, $p < 0.05$. Results are means ± SEMs (n = 10).

3.2 Introducing BDNF-expressing mRNA using polyplex nanomicelles promotes early recovery of olfactory function

An olfactory dysfunction was induced in wild-type BALB/c mice by intraperitoneally injecting methimazole (150 mg/kg body weight) [19]. Methimazole causes cell death and degeneration in the olfactory epithelium, which leads to a transient disturbance in olfactory sensations. These sensations spontaneously shows a gradual recovery over a few weeks, although the olfactory epithelium exhibits metaplasia.

We applied BDNF-expressing mRNA using polyplex nanomicelles to treat this olfactory dysfunction. Olfactory sensations were evaluated with a buried food test to determine the amount of time required for mice to uncover food [29]. As shown in Fig. 4A, intranasal administration of BDNF-expressing mRNA into nose significantly promoted the recovery of olfactory sensations after Day 3, as the average time required to uncover food was remarkably shorter for mice that received BDNF-expressing mRNA in comparison with control mice that received HEPES buffer. Figure 4B shows the percentages of mice that showed curative recovery (could uncover food within 120 s) [29]. All mice that received BDNF-expressing mRNA recovered to the curative level by Day 5, whereas the control mice that received HEPES buffer required 10 days until they all recovered to the curative level.
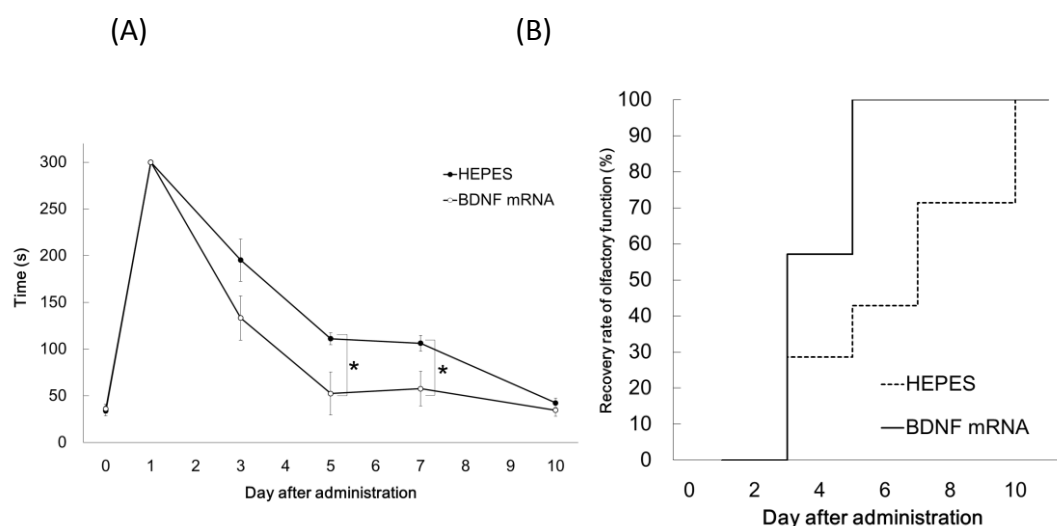
(A)   (B)

Figure 4

Behavioral assessments of olfactory function.

Olfactory sensations were evaluated with a buried food test to determine the amount of

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time required for mice to uncover food. (A) Average time required to uncover food of mice that received BDNF-expressing mRNA (open circle) and control mice that received HEPES buffer (closed circle). (B) Percentages of mice that showed curative recovery (could uncover food within 120 s). Statistical analyses were performed by two-tailed Mann-Whitney U test, *, $p < 0.05$. Results are means ±SEMs (n = 7).

Histopathological examinations of the damaged olfactory epithelium on Day 1 showed that administering BDNF-expressing mRNA maintained the olfactory epithelium in a nearly-normal architecture. A gross analysis of the H&E stained sections of the nasal septa indicated that approximately half of the interior nasal surface area was covered with intact olfactory epithelium that was attached to the lamina propria (Fig. 5A). In contrast, in the control group that received HEPES buffer, the olfactory epithelium was severely sloughed (Fig. 5A) and the area covered by intact olfactory epithelium was significantly reduced to approximately 20% (Fig. 5B).
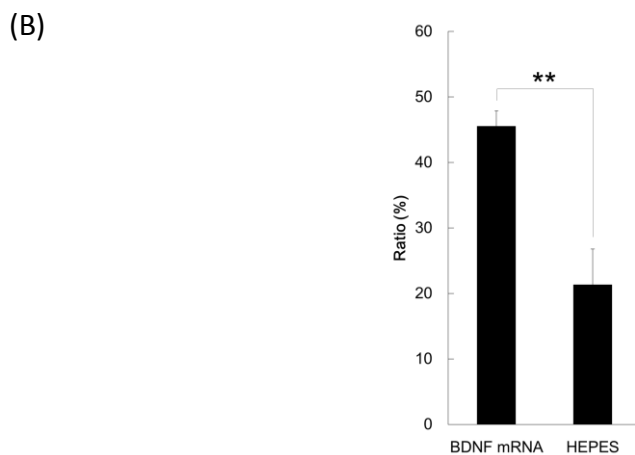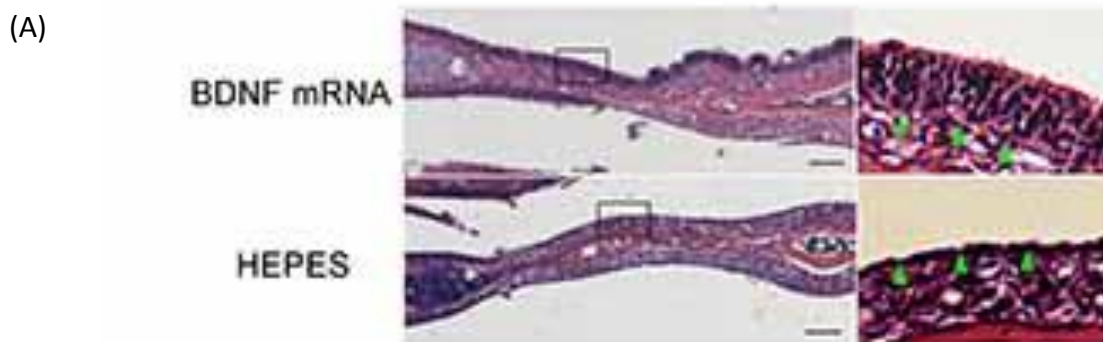
(A)

(B)

Figure 5

Histopathological examination of the damaged olfactory epithelium.

Mice were sacrificed and decapitated 24 h after administering GFP-expressing mRNA. The mandibles were discarded, and the trimmed heads were skinned. Serial sections (thickness, 5 µm) at the level of the anterior end of the olfactory bulb were prepared. These sections were stained with hematoxylin and eosin (H&E). (A) A representative image of a mouse that received BDNF-expressing mRNA or HEPES buffer. Green arrows indicate the basilar membranes. Scale bar: 100 µm. (B) Area ratios that were covered by intact olfactory epithelium. Statistical analyses were performed by two-tailed Student's t-test, **, $p < 0.01$. Results are means ± SEMs (n = 4).
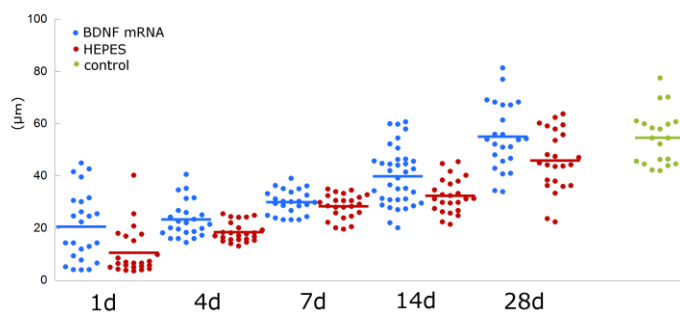
3.3 Introducing BDNF-expressing mRNA using polyplex nanomicelles enables complete regeneration of the olfactory epithelium after drug-induced olfactory dysfunction

To evaluate the long-term effects of BDNF-expressing mRNA on the olfactory epithelium, the thickness of the epithelium was measured via the histological sections by determining the distance between the basilar membrane and the top of the cell layer of the nasal septum. On Day 1, the group that received BDNF-expressing mRNA tended to have a thicker olfactory epithelium than the control group that received HEPES buffer (Fig. 6A, B). This was consistent with the results reported in Fig. 5 regarding the nearly normal architecture of the olfactory epithelium in the BDNF-expressing mRNA treatment group. However, on Days 4 and 7, the mean epithelial thickness was comparable between the groups, presumably due to the enhanced turnover of the epithelium [30]. Further, after Day 14, the treated group showed a higher recovery of epithelium thickness (Fig. 6A). The representative images of the histological sections suggested that the nearly normal architecture of the olfactory epithelium was recovered in the treatment group, whereas in the control group, the epithelium still showed abnormal metaplasia (Fig. 6B).
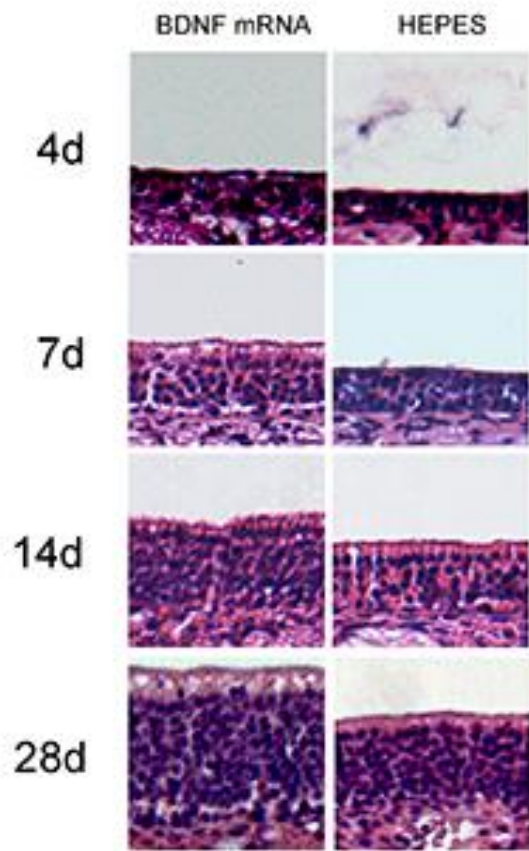
To further confirm the recovery of the olfactory epithelium after administering BDNF-expressing mRNA, we evaluated olfactory marker protein (OMP) which is specifically expressed by mature olfactory neurons [31]. By qPCR analysis of OMP mRNA expression levels in nasal tissues, OMP expression was found to be enhanced, particularly after Day 15, in the treatment group (Fig. 6C). Immunostained sections using

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an anti-OMP antibody (Day 28) indicated that in the treatment group, OMP-positive olfactory neurons were uniformly distributed throughout the nearly normal architecture of the olfactory epithelium (Fig. 6D), whereas in the control group, there were much fewer OMP-positive neurons and they exhibited morphological abnormalities (Fig. 6D). These results strongly suggest that BDNF-expressing mRNA effectively provided a favorable environment to enhance the regeneration of olfactory neurons.
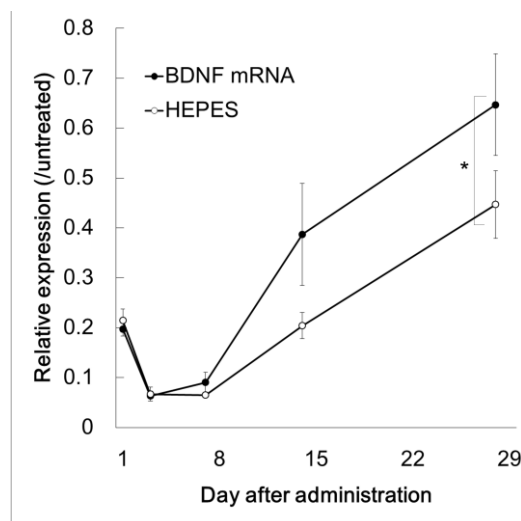
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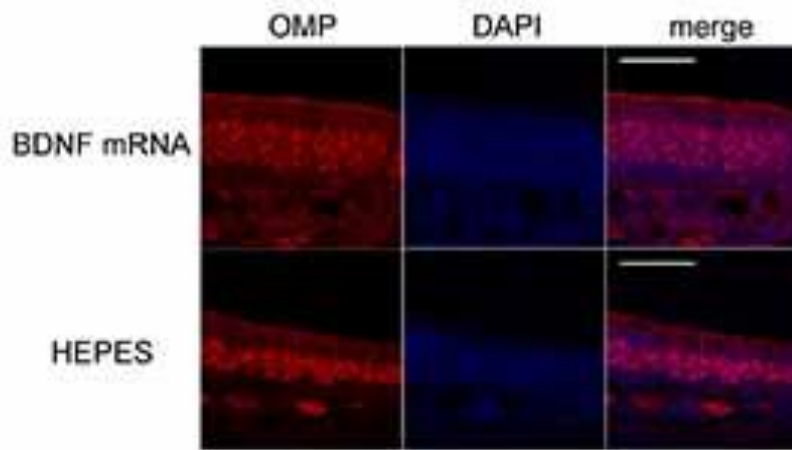

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(D)

Figure 6
Long-term effects of BDNF-expressing mRNA on the olfactory epithelium.
(A) Thickness of olfactory epithelium. The thickness of the epithelium was measured via the histological sections by determining the distance between the basilar membrane and the top of the cell layer of the nasal septum. Blue: Mice that received BDNF-expressing mRNA-loaded polyplex nanomicelles, Red: Those received HEPES buffer, Green: Normal mice that were not injected with methimazole.

(B) Representative images of olfactory epithelium from mice that received BDNF-expressing mRNA-loaded polyplex nanomicelles or HEPES buffer.

(C) OMP mRNA expression levels in nasal tissues expression in the nasal tissue evaluated using real-time quantitative PCR (qPCR). Statistical analyses were performed by two-tailed Student's t-test, *, $p < 0.05$. Results are means ± SEMs (n=7).

(D) Representative images of immunostained sections of olfactory epithelium using an anti-OMP antibody 28 days after administering of BDNF-expressing mRNA-loaded polyplex nanomicelles or HEPES buffer. Scale bar: 50 μm.

4. Discussion

In this study, we demonstrated that intranasal mRNA administration can be used to treat an olfactory nerve dysfunction. The incorporation of mRNA into polyplex nanomicelles provided diffuse protein expression in the lamina propria. BDNF-

expressing mRNA remarkably enhanced the neurological recovery from olfactory dysfunction by repairing the olfactory epithelium to a nearly normal architecture. These results clearly indicate the therapeutic potential of mRNA for neurogenic disorders via sufficient and sustained expression of therapeutic proteins or peptides.

The chief advantage of using mRNA is its competency with non-dividing cells. It is usually difficult to use pDNA transfection for non-dividing cells because of the low efficiency of nuclear import of pDNA [32], however, mRNA does not need to be internalized into the nuclei of these cells, allowing efficient protein expression in the cells. Indeed, in our preliminary study, pDNA exhibited much less transgene expression after intranasal administration, even when using the same polyplex nanomicelle (unpublished data). In contrast, intranasal mRNA administration provided diffuse GFP expression in the lamina propria beneath the basement membrane of epithelial cells (Fig. 2). The lamina propria primarily consists of non-dividing cells, such as nerve and inflammatory cells, interspersed among connective tissue and blood vessels. Although we did not identify individual GFP-positive cells in our histological sections, the diffuse expression of GFP in the lamina propria strongly suggested that mRNA had been successfully introduced into non-dividing cells.

Another advantage of using mRNA is the early onset of protein expression. It has been reported that the protein expression was detectable even 15 min after mRNA transfection, much faster than that after pDNA transfection [33]. In this study, protein expression in the nasal cavity was obvious a few hours after intranasal mRNA administration (Fig. 1A,B), clearly suggesting the rapid onset of protein expression in nasal tissues.

It is notable that the duration of protein expression would tend to be shorter with mRNA considering its rapid degradation [6, 34]. Although little is known about the mechanisms underlying translation control in individual cells, a few studies have reported that the half-lives of mRNA in the cytoplasm were generally in the range of hours [35-37], indicating the very transient manner of protein expression from a single mRNA molecule.

Considering these features of mRNA, the roles of polyplex nanomicelles are not limited to transporting mRNA to target cells by overcoming the barriers in the nasal cavity, including the mucous layer, epithelial membrane and associated junctional barriers. These nanomicelles are also likely to stably retain mRNA in their core, even after they are internalized into target cells, thereby continuously releasing intact mRNA in the cytoplasm. This property enables sustained protein expression in the cells for nearly two days. Thus, it would be expected that therapeutic proteins would be continuously

provided for the nasal tissues by once-daily administration of mRNA-loaded nanomicelles.

These aspects of rapidly-emerging and sustained activity considerably augment the usefulness of mRNA for treating neurological disorders. Indeed, BDNF protein has a half-life of only a few minutes in rat plasma [38], and showed no therapeutic effect on olfactory dysfunction by intranasal administration [39]. Direct administration of BDNF may not attain significant therapeutic outcome for treating neurological disorders because prolonged action in a sustained manner is needed to acquire the neurological recovery. Thus, modification of protein to elongate the half-life, or the use of adjunctive means, such as continuous infusion devices or incorporation into an artificial matrix for controlled drug release, are required because the proteins generally have poor stability under physiological conditions [40, 41]. However, since these devices involve invasive procedures, therapeutic outcomes are not easy to achieve, particularly for neural tissues that require highly delicate manipulations.

From the toxicological standpoints, it is important that intranasal mRNA administration using the nanomicelles induced minimal immune responses, returning to the normal state 24 h after administration. We previously reported that the nanomicelles effectively suppressed mRNA immunogenicity, even when using wild-type mRNA, after administration into the subarachnoid space [14]. Because the nasal cavity is a much more sensitive site for foreign materials, in this study, we used a modified mRNA that exhibited reduced immunogenicity by interfering with the interaction of mRNA with Toll-like receptors [11]. Indeed, even this modified form of naked mRNA triggered strong immune responses after administration into nasal tissues. However, the identical mRNA administered with the nanomicelles induced significantly reduced immune responses (Fig. 3), without any apparent damage to nasal tissues (Fig. 2). Thus, it is reasonable to assume that the nanomicelles are effective to regulate mRNA immunogenicity, further increasing the usefulness of mRNA for therapeutic purposes.

BDNF is a member of the neurotrophin family and its effects include pro-survival activities for neurons under various pathological conditions [42-45], and synaptic repair capacity to enhance synaptic transmission, facilitate synaptic plasticity and promote synaptic growth [46, 47]. In agreement with its expected functions, the effects of BDNF demonstrated in this study appeared to include two phases. The first was a neuroprotective effect against methimazole-induced damage, as indicated by the well preserved olfactory epithelial structure and early recovery of olfactory sensations within a few days after introducing BDNF-expressing mRNA. Although it was difficult to directly determine the amount of BDNF in the nasal tissue of small animals like mice by ELISA, a

therapeutic quantity of BDNF protein may be provided in a sustained manner in the nasal cavity through the expression of BDNF-coded mRNA for 5 consecutive days. The latter phase of BDNF effects involved enhancing the regeneration of mature olfactory neurons with significant differences from the untreated controls, as reflected by the increased thickness of the olfactory epithelium and enhanced OMP signals (Fig. 6). After neurological damage, OMP signals gradually decrease for a week during cell dropout from the epithelium, then shows a slow recovery in parallel with the mitoses of basal cells [48]. In this study, it is interesting that these effects were observed for a few weeks after mRNA administration, although the period of administration was limited to 5 days. Thus, the supply of exogenous BDNF protein to nasal tissues was likely to be terminated approximately a week after inducing an olfactory dysfunction. A reasonable speculation is that BDNF expressed by mRNA not only stimulated the olfactory neurons for enhanced regeneration, but also effectively ameliorated the pathological conditions in nasal tissues to be suitable for nerve regeneration.

In conclusion, we used mRNA to treat olfactory dysfunction by in vivo intranasal administration using polyplex nanomicelles. mRNA provided efficient, sustained protein expression for nasal tissues, particularly in the lamina propria. BDNF-expressing mRNA remarkably enhanced the neurological recovery of olfactory function along with repairing the olfactory epithelium to a nearly normal architecture. These results indicate the feasibility and safety of using mRNA, and provide a novel strategy of mRNA-based therapy for neurogenic disorders.

Acknowledgements

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Figure legends

Figure 1
Bioluminescence after intranasally administering luciferase-expressing mRNA.
(A) Bioluminescence images obtained by an IVIS Imaging System 4 h after administering luciferase-expressing mRNA-loaded polyplex nanomicelles (upper) and an equal quantity of naked mRNA (lower).
(B) Time course of bioluminescence after intranasally administering mRNA using polyplex nanomicelles (closed circle) and naked mRNA (open circle). Statistical analyses were performed by two-tailed Student's t-test, *** $p < 0.001$, ** $p < 0.01$. RLU; relative luminescence units. Results are means ± SEMs (n = 4).

Figure 2
Histological analysis after intranasally administering GFP-expressing mRNA.
Mice were sacrificed and decapitated 24 h after administering GFP-expressing mRNA. (A) GFP expression visualized by immunostaining using an anti-GFP monoclonal antibody. (B) Cell nuclei stained by Hoechst. (C) Merged image. GFP-positive staining was widely observed in the lamina propria (arrowheads), but not in nasal septal cartilages and bones (asterisk). Scale bar: 50 μm.

Figure 3
Induction of proinflammatory cytokine genes after intranasal mRNA administration.
Proinflammatory gene mRNA expression levels were evaluated 4 and 24 h after mRNA administration using real-time quantitative PCR (qPCR) by measuring the mRNA expression levels of genes encoding tumor necrosis factor (TNF)-α (A) and interleukin (IL)-6 (B) in nasal tissues administration of mRNA into the nasal tissue. mRNA was administered using polyplex nanomicelle (open bar) or in the form of naked mRNA (closed bar). Statistical analyses were performed by two-tailed Student's t-test, *, $p < 0.05$. Results are means ± SEMs (n = 10).

Figure 4
Behavioral assessments of olfactory function.
Olfactory sensations were evaluated with a buried food test to determine the amount of time required for mice to uncover food. (A) Average time required to uncover food of mice that received BDNF-expressing mRNA (open circle) and control mice that received HEPES buffer (closed circle). (B) Percentages of mice that showed curative recovery

(could uncover food within 120 s). Statistical analyses were performed by two-tailed Mann-Whitney U test, *, p < 0.05. Results are means ±SEMs (n = 7).

Figure 5

Histopathological examination of the damaged olfactory epithelium.

Mice were sacrificed and decapitated 24 h after administering GFP-expressing mRNA. The mandibles were discarded, and the trimmed heads were skinned. Serial sections (thickness, 5 μm) at the level of the anterior end of the olfactory bulb were prepared. These sections were stained with hematoxylin and eosin (H&E). (A) A representative image of a mouse that received BDNF-expressing mRNA or HEPES buffer. Green arrows indicate the basilar membranes. Scale bar: 100 μm. (B) Area ratios that were covered by intact olfactory epithelium. Statistical analyses were performed by two-tailed Student's t-test, **, p < 0.01. Results are means ± SEMs (n = 4).

Figure 6

Long-term effects of BDNF-expressing mRNA on the olfactory epithelium.

(A) Thickness of olfactory epithelium. The thickness of the epithelium was measured via the histological sections by determining the distance between the basilar membrane and the top of the cell layer of the nasal septum. Blue: Mice that received BDNF-expressing mRNA-loaded polyplex nanomicelles, Red: Those received HEPES buffer, Green: Normal mice that were not injected with methimazole.

(B) Representative images of olfactory epithelium from mice that received BDNF-expressing mRNA-loaded polyplex nanomicelles or HEPES buffer.

(C) OMP mRNA expression levels in nasal tissues expression in the nasal tissue evaluated using real-time quantitative PCR (qPCR). Statistical analyses were performed by two-tailed Student's t-test, *, p < 0.05. Results are means ± SEMs (n=7).

(D) Representative images of immunostained sections of olfactory epithelium using an anti-OMP antibody 28 days after administering of BDNF-expressing mRNA-loaded polyplex nanomicelles or HEPES buffer. Scale bar: 50 μm.