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Transfer of small interfering RNA by single-cell electroporation in cerebellar cell cultures

Masahiko Tanaka [a,*], Yuchio Yanagawa [b], Naohide Hirashima [a]

[a] Department of Cellular Biophysics, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan
[b] Department of Genetic and Behavioral Neuroscience, Graduate School of Medicine, Gunma University, Maebashi 371-8511, Japan

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ABSTRACT

RNA interference (RNAi) is a powerful means to investigate functions of genes involved in neuronal differentiation and degeneration. In contrast to widely used methods for introducing small interfering RNA (siRNA) into cells, recently developed single-cell electroporation has enabled transfer of siRNA into single and identified cells. To explore the availability of single-cell electroporation of siRNA in detail, we introduced siRNA against green fluorescent protein (GFP) into GFP-expressing Golgi and Purkinje cells in cerebellar cell cultures by single-cell electroporation using micropipettes. The temporal changes in the intensity of GFP fluorescence in the same electroporated cells were monitored in real-time up to 4 days after electroporation. Several parameters, including tip diameter and resistance of micropipettes, concentrations of siRNA and a fluorescent dye marker, voltage and time of pulses, were optimized to maximize both the efficacy of RNAi and the viability of the electroporated cells. Under the optimal conditions, transfer of GFP siRNA significantly reduced GFP fluorescence in the electroporated cells, whereas that of negative control siRNA had no effects. GFP siRNA was more efficient in Purkinje cells than in Golgi cells. The electroporated Purkinje cells were normal in their morphology, including elaborated dendrites. Thus, the single-cell electroporation of siRNA could be a simple but effective tool for silencing gene expression in individual cells in neuronal primary cultures. In addition, both gene-silencing and off-target effects of siRNA introduced by this method may differ between neuronal cell types, and the parameters of single-cell electroporation should be optimized in each cell type.

1. Introduction

RNA interference (RNAi) is a powerful means to investigate functions of genes involved in neuronal differentiation and degeneration. Among several methods for RNAi, small interfering RNA (siRNA)-based RNAi has the advantage of simplicity, although the success of this method largely depends on the efficiency of siRNA introduction. Widely used methods for introducing siRNA into neuronal cells are lipofection, bulk electroporation, gene gun biolistics and viral infection. However, it is impossible to introduce siRNA into identical and single cells by these methods.

Single-cell electroporation is a recently developed method to introduce polar and charged molecules such as dyes, drugs, peptides, proteins and nucleic acids (Olofsson et al., 2003). In contrast to traditional bulk electroporation using large plane electrodes, single-cell electroporation uses electrolyte-filled capillaries (Nolkrantz et al., 2001), micropipettes (patch-clamp electrodes) (Haas et al., 2001; Rae and Levis, 2002; Rathenberg et al., 2003; Lovell et al., 2006) or chip structures (Huang and Rubinsky, 1999; Khine et al., 2005; Vassanelli et al., 2008) to transfer the molecules of interest into single cells. By this method, it is possible to transfer siRNA into microscopically identified cells (Boudes et al., 2008). This enables us to inhibit target genes in specific cells within a heterogeneous cell population. This feature of single-cell electroporation is advantageous, especially in investigations of the nervous system because the nervous system is composed of various types of cells.

In spite of its prospective usefulness in neuroscience, many aspects of availability of siRNA single-cell electroporation remain to be elucidated. For instance, what are the effective concentrations of siRNA necessary for specific gene silencing? Does neuronal differentiation such as dendrite formation proceed normally after single-cell electroporation? Is it similarly applicable to different types of neurons? Do the gene-silencing and off-target effects occur at the same level between different types of neurons?

To explore these aspects, we introduced siRNA into cerebellar cell cultures by single-cell electroporation using micropipettes. Cerebellar cell cultures were prepared from glutamate decarboxylase 67-green fluorescent protein (GAD67-GFP) knock-in mice, in which GABAergic neurons, including cerebellar Purkinje cells and inhibitory interneurons express GFP (Tamamaki et al., 2003). We

* Corresponding author. Tel.: +81 52 836 3412; fax: +81 52 836 3414.
E-mail address: mtanaka@phar.nagoya-cu.ac.jp (M. Tanaka).

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introduced siRNA against GFP into Golgi and Purkinje cells in the cultures and monitored the temporal changes in the intensity of GFP fluorescence in the same electroporated cells up to 4 days after electroporation by repeated observation and imaging. Several parameters, including tip diameter and resistance of micropipettes, concentrations of siRNA and a fluorescent dye marker, voltage and time of pulses, were optimized to maximize both the efficacy of RNAi and the viability of the electroporated cells. The results obtained showed that single-cell electroporation of siRNA can be a simple but effective tool for silencing gene expression in individual cells in neuronal primary cultures. Furthermore, we examined concentration-dependencies of both specific gene-silencing and non-specific off-target effects of siRNA introduced by this method, showing that these effects differed between neuronal cell types.

2. Materials and methods

2.1. Animals

In this study, we used GAD67-GFP (Δneo) mice, in which GFP is selectively expressed under the control of the endogenous GAD67 gene promoter as described previously (Tamamaki et al., 2003; Tanaka et al., 2006). These transgenic mice were called GAD67-GFP knock-in mice. The mice were maintained with a genetic background of C57BL/6 at our animal facility. Animal experiments were carried out in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals revised 1996, and approved by the animal care and use committee at Nagoya City University. All efforts were made to minimize the number of animals used and their suffering.

2.2. Dissociated cell culture

The method of cerebellar dissociated cell culture "derived from GAD67-GFP knock-in mice" was largely described previously (Tanaka et al., 2006). We mated a mouse heterozygous for the GAD67-GFP allele ($GAD67^{GFP/+}$) with a wild-type C57BL/6 mouse, and used both $GAD67^{GFP/+}$ and $GAD67^{+/+}$ pups obtained from the breeding pair for the following two reasons. First, seven or more pups per experiment were needed to maintain the viability of Purkinje cells in dissociated cell cultures. Second, the presence of GFP-negative cells from $GAD67^{+/+}$ pups enabled us to decrease the density of GFP-expressing cells in dissociated cell cultures, thus facilitating our identification of the electroporated cells in repeated observations. We usually used seven to nine cerebella of neonatal mice to prepare nine to eleven dishes of dissociated cell cultures for one experiment.

The cerebella of neonatal (postnatal day 0 or 1) GAD67-GFP knock-in mice were dissected in a 1:1 mixture of phosphate-buffered saline (PBS) and Eagle's basal medium with Hank's salts (dissection medium). After removal of the meninges and mincing with a pair of scalpel blades (No. 11; Feather, Osaka, Japan), specimens were incubated for 30 min at 37 °C in 8 U/ml papain (Worthington, Lakewood, NJ, USA), 20 U/ml DNase I (Sigma, St. Louis, MO, USA) and 5 mM cysteine-HCl in PBS. After adding fetal bovine serum to stop the proteolytic reaction and washing by centrifugation at 700 rpm ($80 \times g$) in fresh dissection medium, the tissues were triturated with a Gilson-compatible pipette tip (blue type; Watson/Nippon Genetics, Tokyo, Japan) and suspended in a serum-free culture medium composed of Eagle's basal medium with Earle's salts supplemented with 1 mg/ml bovine serum albumin, 5.6 g/l D-glucose, 3 mM L-glutamine, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 30 nM sodium selenite, 20 nM progesterone and 1 mM sodium pyruvate. After centrifugation at 1000 rpm ($170 \times g$), the cell pellet was resuspended in serum-free culture medium and strained through a 40-μm nylon mesh filter (Falcon 2340; Becton Dickinson, Franklin Lakes, USA). Then, the cell suspension, at a concentration of approximately 5×10^6 cells per ml, was plated on a plastic-bottom dish (μ-Dish; plating area, 21 mm in diameter; ibidi, Nippon Genetics, Tokyo, Japan) coated with poly-L-lysine (MW > 300,000; Sigma) at a density of $1.5–1.8 \times 10^5$ cells per cm^2. After 1.5 h incubation, 0.5 ml of the Sumitomo Nerve-Cell Culture Medium (Sumitomo Bakelite, Tokyo, Japan) was added to each dish. The cultures were incubated at 37 °C in 5% CO_2/95% air.

2.3. siRNA

The negative control and GFP siRNAs were purchased from Ambion/Applied Biosystems (Austin, TX, USA). The siRNAs were diluted into a solution containing 150 mM potassium methanesulfonate and 5 mM Hepes (pH 7.2). Alexa Fluor 594 (AF594; Molecular Probes/Invitrogen, Eugene, OR, USA) was added to the pipette to monitor the electroporation and visualize the electroporated cells.

2.4. Single-cell electroporation of siRNA

Single-cell electroporation was performed in GFP-positive Golgi and Purkinje cells at 9–11 or 15–16 days *in vitro* (DIV). Equipment for single-cell electroporation is shown in Fig. 1. The dissociated cell cultures were transferred to the stage of an inverted microscope equipped with epifluorescence (Axiovert; Zeiss, Oberkochen, Germany). Golgi and Purkinje cells were identified by their morphology (see Section 3). Micropipettes were pulled on a micropipette puller (PB-7; Narishige, Tokyo, Japan) using thin-walled 1.2 mm o.d. filament glass capillary (GD-1.2; Narishige). Micropipette tips (roughly 0.5 μm in diameter and 30–40 MΩ resistance) were filled with 3 μl of siRNA/AF594. A silver wire electrode (cathode) was placed in the micropipette, whereas a ground electrode (anode) was placed in

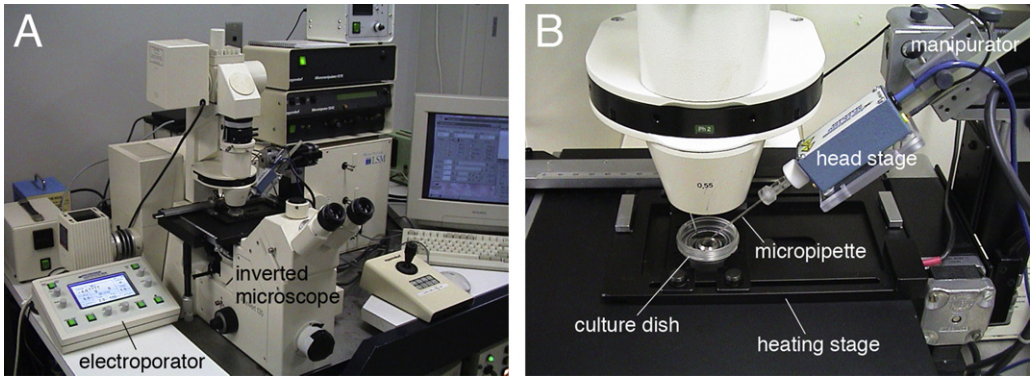

Fig. 1. The single-cell electroporation set-up.