RNAi-Induced Gene Silencing by Local Electroporation in Targeting Brain Region

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Akaneya, Yukio, Bin Jiang, and Tadaharu Tsumoto. RNAi-induced gene silencing by local electroporation in targeting brain region. J Neurophysiol 93: 594–602, 2005; doi:10.1152/jn.00161.2004. Genetic manipulation for “knockout” (KO) is a useful tool for characterizing a target gene. However, its shortcomings that need to be overcome hinder its easy and ready usage in ordinary laboratories. Here we describe a knockdown technique termed the RNA interference (RNAi)-induced gene silencing by local electroporation (RISLE). Small interfering RNA (siRNA) introduction by electroporation into a specific brain region results in a marked reduction in the expression levels of both the mRNA and protein of the target genes such as GluR2 and Cox-1 without affecting the expression levels of proteins other than that of the target protein or causing pathological changes in the target tissues. The effective electrical pulses are relatively weak, consisting of a short strong pulse and a weak long pulse applied in tandem. RISLE can knock down a gene at the target region, for example, the visual cortex and the CA1 region of the hippocampus, without affecting other regions. Moreover, the knockdown models constructed using this technique have physiological functions consistent with previous findings, that is, glutamate release from presynaptic sites, long-term potentiation (LTP), and long-term depression (LTD). These results suggest that this technique is applicable and characterized by spatial flexibility, temporal accessibility, and ease of establishment of knockdown models. The intactness of the tissue subjected to RISLE is due to the weak electrical pulses applied and the limited area of gene silencing. Thus RISLE may be applicable to disease therapy in the future.

INTRODUCTION

The most prominent feature of genetic manipulation for knockout (KO) is the specificity for a target gene the expression of which is blocked. However, conventional KO techniques have some problems: the unpredictable influences of the knocked-out protein on other proteins that may sequentially induce unpredictable influences on other proteins particularly during embryonic and developmental stages; the frequent occurrence of death of animals before birth or during early development, resulting in reduced opportunity of execution of experiments; the limitation of the knocked-out region (in most cases, the whole body); and animal constraints for experiments (in most cases, mice). Recently developed conditionally KO animals have overcome these problems to some degree (Tsien et al. 1996). However, this technique has problems such as the enormous expense, labor, and time required and difficulty in establishing such animals. Therefore these conventional KO techniques have been commonly used for the functional analysis of a target protein by many researchers mainly because of the specificity of targeting that prevents the presumable nonspecific action induced by, for example, pharmacological agents.

As an alternative method of inhibiting target gene expression, RNA interference (RNAi) is a phenomenon in which 21–23 nucleotides (nt) of RNA, termed siRNA, inhibit the target gene expression (Fire et al. 1998). In respect to the specificity for a target gene and the efficacy of RNAi maintenance, the method using siRNA has great advantages over that using conventional antisense oligonucleotides in knocking down the target because siRNAs have more effective knockdown at its targets at lower concentration than antisense oligonucleotides (Brantl 2002).

For introducing exogenous molecules such as proteins, RNA, and DNA into cells, electroporation is a convenient and efficient method (Neumann et al. 1999). The mechanisms underlying its efficacy was suggested to comprise two steps; first electrical shocks applied to the membrane produce pores the lifetime of which is tens of seconds and then negatively charged external molecules enter into the cytosol by electrophoresis. In most cases, the targets of in vivo electroporation have been whole organs such as the liver and brain limited to chick and mouse embryonic stages (Agarwala et al. 2001; Fukuchi-Shimogori and Grove 2003; Inoue and Krumlauf 2001; Sukharev et al. 1992). Electroporation at embryonic stages possibly affects the fate of proteins other than the target protein as mentioned in the preceding text.

To overcome the above-mentioned problems in systemic knockout manipulation, we have developed a novel method by which siRNA is introduced into a restricted target brain region by electroporation using two needle electrodes. This method is termed RNAi-induced gene silencing by local electroporation, RISLE.

METHODS

Animals and anesthesia

Sprague Dawley (SD) rats, ranging in age from postnatal days 15 to 20 (P15–P20), were used. The animals were raised with water and food ad libitum and kept on 12-h light/dark cycle. The experimental procedures were in accordance with the regulations of the Animal Care Committee of Osaka University Graduate School of Medicine. The rats were anesthetized with an intraperitoneal injection of pento-
barbital sodium (Nembutal, Abbot Laboratories, North Chicago, IL) at 20–30 mg/kg and placed in a stereotaxic frame. Anesthesia was maintained throughout experiments by injecting a supplemental dose of pentobarbital sodium (Nembutal, 0.5–1 mg/h) if necessary to keep the level of anesthesia. Rectal temperature was maintained at 37 ± 0.5°C with a servo-heating pad. An appropriate dose of atropine sulfate (0.5 mg/kg) was injected subcutaneously to reduce respiratory secretions, and heart rate was monitored continuously to ensure preparation stability.

Preparation of siRNA

To design the target siRNA sequences of iRGluR2 and iRCox-1 and ascertain the uniqueness of these sequences, the NCBI library and NCBI nucleotide BLAST were referred to. Four sites were selected for each target, and the following oligonucleotide templates were used for iRGluR2a (antisense): AAGGGGCCTGATACAGAACGCTGTCTC; iRGluR2a (sense): AATATTCTTGATAGGCCTCCCTGTCTC; iRGluR2b (antisense): AAAGCCCTTGATAGGCTGTCTC; iRGluR2b (sense): AACCTACAACACAGCACATGACCTGTCTC; iRCox-1a (antisense): AAGTACCCAGCCCATGAGTACCCTGTCTC; iRCox-1a (sense): AACCTACAACACAGCACATGACCTGTCTC; iRCox-1b (antisense): AAGTACTCATGGGCTGGGTACCCTGTCTC; iRCox-1b (sense): AACCTACAACACAGCACATGACCTGTCTC; iRCox-1c (antisense): AAGTACCGCAAGCGGCCTGTCTC; iRCox-1c (sense): AACCTACAACACAGCACATGACCTGTCTC; iRCox-1d (antisense): AACCTACAACACAGCACATGACCTGTCTC; iRCox-1d (sense): AACCTACAACACAGCACATGACCTGTCTC; iRGluR2c (antisense): AAGCTGTTCTGGATGCGCTGTCTC; iRGluR2c (sense): AAGCTGTTCTGGATGCGCTGTCTC; iRGluR2d (antisense): AACAGCA-GCTCCCCTGTCTC; iRGluR2d (sense): AAGCTGTTCTGGATGCGCTGTCTC; iRGluR2b (antisense): AAATTGGTGACTGCGAAACTGTACTATCCCTGAGATCCCTGTCTC; iRGluR2b (sense): AACAGCA-GCTCCCCTGTCTC; iRGluR2a (antisense): AATATTCTTGATAGGCCTCCCTGTCTC; iRGluR2a (sense): AATATTCTTGATAGGCCTCCCTGTCTC

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Injection of siRNA and electroporation

P15–P20 SD rats were used in the experiments. After the rats were anesthetized, three adjacent perforations of 2-mm diam were made on the skull using a dental drill (Fig. 1A); the middle hole was for the injection of siRNA and the others for the insertion of electrodes for electroporation. The stereotaxic coordinates for injections were as follows: for the visual cortex, 4.8–5.2 mm posterior to the bregma, 1.5–2.0 mm lateral to the midline, and 0.5–0.6 mm in depth; and for the hippocampus (CA1 region), 3.8–4.0 mm posterior to the bregma, 1.5–2.0 mm lateral to the midline, and 2.5–2.8 mm in depth. Solutions containing siRNAs were applied to the target region through the middle pore of the skull with RNase-free polyethylene tubes connected to a Hamilton syringe between the siRNA injection site with 5-mm width of film with a noncoated 2-mm tip (Fig. 1B). This coating minimized damage to the tissue and was adjustable depending on the volume or shape of the target region. These electrodes at 5-mm lateral to the midline, and 2.5–2.8 mm in depth, Solutions containing siRNAs were applied to the target region through the middle pore of the skull with RNase-free polyethylene tubes connected to a Hamilton syringe through an isolator (SS-201J, Nihon Koden). After the injection of siRNA and the others for the insertion of electrodes for stimulation electrodes for electroporation, a pair of parallel stimulation electrodes. The tandem pulses, the intervals of which were fixed to 10 ms, consist of the first pulse of high-voltage and short-duration and the 2nd pulse with a low voltage and a long duration.

depends on the paradigms of electric pulses, the combination of a preceding poring pulse of high-voltage and short-duration and a following driving pulse of low voltage and long duration in tandem was employed in the present study (Fig. 1C). It is expected that the poring pulse forms pores in the plasma membrane, and the driving pulse makes extracellular molecules enter into the cytosol.

Western blot analysis

Under a microscope, the tissue including the siRNA injection site and the contralateral control tissue were cut into 2-mm cubes in cold PBS containing heparin on ice. The tissues were homogenized with 15 strokes in 300 µl of lysis buffer containing 25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, adequate concentration of complete protein inhibitor (Roche), and 0.7 µg/ml pepstatin in a 2-ml glass syringe on ice and then incubated with shaking for 30 min at 4°C, followed by centrifugation at 15,600 g for 10 min at 4°C. The supernatant was then used for Western blot analysis, and one portion of the supernatant was used for protein level determination using the Bio-Rad protein assay (Bio-Rad). After boiling for 3 min, the supernatants in 62.5 mM Tris-HCl (pH6.8), 19% glycerol, 2.3% SDS, 0.01% bromphenol blue, and 0.5% β-mercaptoethanol were loaded to SDS-PAGE for separation, followed by the transfer of separated products to a nitrocellulose membrane. After incubation in PBS with 5% skimmed milk and 0.1% Tween 20 (T-PBS), the membrane was incubated with the primary antibody in T-PBS with shaking for 2 h. The dilutions of the primary antibodies used were as follows: anti-GluR1 antibody (Chemicon), 1:500; anti-GluR2 antibody (Chemicon), 1:500; anti-NR1 antibody (BD Biosciences), 1:500; anti-Cox-1 antibody (Cayman), 1:500; anti-Cox-2 antibody (Cayman), 1:500; anti-GRIP1 antibody (C-20)(Santa Cruz), 1: 200; anti-GRIP2 antibody (K-17) (Santa Cruz), 1:200; and anti-α-tubulin antibody (Sigma), 1:15,000. After washing three times with T-PBS, the membrane was exposed to the anti-mouse (Amersham Biosciences, Piscataway, NJ).
taway, NJ), anti-rabbit (Amersham Biosciences), or anti-goat (Santa Cruz) peroxidase-conjugated IgG secondary antibody (0.7 μg/ml) or 1 h. After washing four times with T-PBS, the membrane was developed using ECL Western blotting detector reagents (Amersham Biosciences).

For Western blot and quantitative real-time PCR analyses, the expressions were normalized to the contralateral side of the RISLE-subjected tissue (electroporation-subjected side without siRNA) except for the experiment in Fig. 3E. The analyses were performed 5–7 days after electroporation unless otherwise mentioned.

**Preparation for cDNA and quantitative real-time PCR**

The tissues around the RISLE and contralateral sites were removed and stored in RNA Later stabilization reagent (Quiagen) at −80°C. The tissues were cut into pieces with scalpels; the pieces were put in a cold lysis buffer, and then passed through a 20-gauge needle. Using an RNeasy minikit (Quiagen), total RNA was prepared using these lysates according to the manufacturer’s instructions.

To construct cDNA, the template RNA was incubated with 4 μl reaction Omniscript reverse transcriptase (Quiagen), dNTP mix (5 mM, for each dNTP), 1 μM oligo-dT primer (Roche), and 10 μl reaction RNase inhibitor for 1 h at 37°C. Then to inactivate reverse transcriptase, the reaction mixture was incubated at 93°C for 5 min, followed by rapid cooling on ice.

For quantitative real-time PCR, the primers and TaqMan probes for GluR2 and Cox-1 were designed and synthesized according to Assay-by-Design (Applied Biosystems, ABI). The sequences used were as follows: TaqMan probe for Cox-1: CCGCATGCATGGAAT-TCAGTG; 5’ primer for Cox-1: CGAGCCCAGTTCCAGTATCG; 3’ primer for Cox-1: TGAACGGATGCCAGTGATAGAG; TaqMan probe for GluR2: TGGATCAGGGTCCATGGGTGAG; 5’ primer for GluR2: ATGGGAAGGTGCTGATATC; 3’ primer for GluR2: AGTGTAGCTGGTGCTGTTGA.

The TaqMan probes have FAM as the reporter at the 5’ end and TAMRA as the quencher at the 3’ end. For endogenous control, TaqMan rodent GAPDH control reagents (ABI) were used. The template cDNA in the TaqMan Universal PCR Master Mix (ABI) was amplified using an ABI PRISM 7900HT sequence detection system.

The conditions for the PCR run were as follows: 50°C for 2 min and 95°C for 10 min; 95°C for 15 s followed by 60°C for 1 min for 40 cycles; and storage at 25°C. The data were normalized to the data amplified using an ABI PRISM 7900HT sequence detection system.

For stimulating the lateral geniculate nucleus (LGN), a bipolar stimulation electrode was inserted at the stereotaxic coordinates of 4.0 mm posterior to the bregma and 3.5–4.0 mm lateral to the midline. The tip of the electrode was adjusted to its most suitable depth at which maximal field responses to flashes of light presented to the eye contralateral to the LGN are observed. For the monopolar recording of cortical field potentials near the RISLE area in visual cortex, a single-barrel borosilicate glass micropipette filled with 3 M NaCl was placed 5.8–6.0 mm posterior to bregma, 4.0–4.2 mm lateral to the midline and in layer II/III of the cortex by lowering it vertically 0.5 mm below the pial surface.

Field potentials were evoked by single-shock stimuli of 0.2-ms duration at an intensity of 0.2–0.9 mA as described previously (Jiang et al. 2001, 2003). The evoked potentials were amplified and filtered at 0.1–3 kHz, digitized at 20 kHz, and stored using PowerLab software (AD Instruments). To determine the intensity of stimulation in the experiments, a full input-output curve was plotted. The intensity yielding amplitude of potentials at 50–60% of the maximal potentials was used in the experiments.

**Electrophysiology in vitro**

The experiments were carried out as previously described (Akaneya et al. 1996, 1997). After anesthetizing the rats, the region of the visual cortex between two electroporation scars was quickly removed and placed in chilled ACFC saturated with 95% O2-5% CO2. The tissues were sectioned into coronal slices of 400 μm thickness with a rotor slicer (DTY7000, Dosaka), and the sections were incubated with ACFS including 5 μg/ml PI for 30 min. After washing, images of the sections were taken through a confocal microscope.

**Analysis for OAS1 expression**

RISLE was performed for the visual cortex with iRGluR2 or iRcox-1. The preparation for cDNA and the subsequent quantitative real-time PCR for OAS1 were carried out with Assay on demand kit (Applied Bioscience) as described in the preceding text.

**Preparation of synaptoneurosomes and measurement of glutamate release**

The visual cortex on the RISLE and contralateral sides were gently homogenized on ice in a glass homogenizer containing a solution with (in mM) 125 NaCl, 5 KCl, 1 MgCl2, 1.2 Na2HPO4, 10 glucose, and 20 HEPES/NaOH, pH 7.4. These lysis solutions were filtered sequentially through two layers of 100-μm fine nylon mesh and a 5-μm filter followed by centrifugation at 1,000 g at 4°C for 10 min.

Glutamate release from synaptoneurosomes was measured using an enzyme-coupled fluorometric assay with some modifications (Sihra et al. 1992). The suspension of synaptoneurosomes was stirred at 37°C, followed by the addition of a solution (final concentration) of NADP (1 mM), glutamate dehydrogenase (50 units/ml), and CaCl2 (1 mM). After 3 min, KCl (30 mM) or brain-derived neurotrophic factor (BDNF; 200 ng/ml) was added to the solution. Fluorescence was monitored using a spectrofluorimeter at 340 nm (excitation) and 460 nm (emission).

**Immunohistochemistry**

After anesthetizing, the rats were transcardially perfused with 4% paraformaldehyde in PBS, followed by incubation with 10% sucrose in PBS. The tissues were sectioned with a freezing microtome (JUNG KM2000, Leica) at 60–80 μm thickness, followed by permeabilization in PBS with 0.2% Triton X-100 for 5 min. After blocking with 5% skimmed milk in PBS, the sections were incubated with the anti-GluR2 (1: 200; Chemicon) or Cox-1 (1: 100; Cayman) antibody at 4°C for 24–48 h. The immunohistochemical reaction was developed using the Vectastain ABC kit (Vector Laboratory, Burlingame, CA).

**Estimation of cell death**

The injection sites of glutamate and actinomycin D (AcD) were stereotaxically determined in the visual cortex. The electroporation stimulation electrodes were inserted on the contralateral side, and focal electroporation was performed with iRGluR1 or iRcox-1. After 5 days, for apoptosis detection, fixation, sectioning, permeabilization, and visualization were performed as described in the immunohistochemistry section. Using an in situ cell-death-detection kit, POD (Roche), sections on a cover glass were incubated with fluorescein-12-dUTP at 37°C for 1 h. After washing, the sections were incubated with the anti-fluorescein antibody conjugated with HRP. For propium iodide (PI) uptake determination, the visual cortex including the injection site was removed and placed in artificial cerebrospinal fluid (ACSF) saturated with 95% O2-5% CO2. The composition of ACSF was as follows (in mM): 124 NaCl, 5 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, and 10 glucose. The tissues were sectioned into coronal slices of 400 μm thickness with a rotor slicer (DTY7000, Dosaka), and the sections were incubated with ACSF including 5 μg/ml PI for 30 min. After washing, images of the sections were taken through a confocal microscope.
RESULTS

Determination of values of parameters of electrical pulses

For RISLE, we used two electrical pulses, the preceding poring pulse and the following driving pulse (Fig. 1C) and determined the most effective values of parameters of electrical pulses for the knockdown of GluR2, that is, the voltage and duration of the poring pulse ($V_d$ and $D_p$, respectively, in Fig. 1C) and those of the driving pulse ($V_a$ and $D_a$, respectively, in Fig. 1C). In preliminary experiments, we assumed that the most effective values are as follows; $V_p > 100$ V/cm, $D_p \approx 1$ ms, $V_d > 2$ V/cm, and $D_d \approx 2$ s. First, we altered the voltage of the poring pulse ($V_p$) at fixed values of the other parameters ($D_p = 1$ ms, $V_d = 2$ V/cm, $D_d = 2$ s). The results showed that 100 and 200 V/cm can reduce GluR2 expression level most effectively (the value decreased to 31.2% of control; Fig. 2A), while 10 V/cm was slightly effective (the value decreased to 72.4% of control; Fig. 2A). Regarding the investigation at fixed values of parameters ($V_p = 100$ V/cm, $V_d = 2$ V/cm, $D_d = 2$ s), the effects of poring pulse duration ($D_p$) seemed not so much different in the duration examined, but 2 ms appears to be the most effective (the value decreased to 22.5% of control; Fig. 2B). Next, the voltage ($V_a$) and duration ($D_a$) of the driving pulse were changed at a fixed poring pulse ($V_p = 100$ V/cm, $D_p = 2$ ms). For $V_a$ values of >2 V/cm are the most effective (27.4 ± 17.4 at 2 V/cm, $n = 4$, $P < 0.005$ in comparison with the control by ANOVA; Fig. 2C) at a fixed $D_a$ of 2 s. For $D_a$ at a fixed $V_a$ of 2 V/cm, values of >2 s are the most effective (the value decreased to 32.7% of control; Fig. 2D), but the effect of 0.5-s duration is not significant (the value decreased to 87.5% of control; Fig. 2D). Thereafter $V_p = 100$ V/cm, $D_p = 1$ ms, $V_d = 2$ V/cm, and $D_d = 2$ s were used throughout the experiments.

Next we checked whether only application of siRNA or only electroporation may affect the expression of targets independently. The direct injections of iRGluR2 or iRCox-1 into the visual cortex without electroporation and the subjection of electroporation to the visual cortex failed in the knockdown of the targets ($n = 3–4$; Fig. 2E). These results suggest that siRNAs cannot enter into cells without electroporation and that electroporation by itself cannot affect the expression of targets.

Time course of effect of RISLE on mRNA expression

To test the effect of RISLE on the level of mRNA with time, we performed quantitative real-time PCR analysis to determine the level of cDNA, which was reverse-transcribed from the mRNA obtained from a portion of tissues distributed between two electrodes. For the siRNA of iRGluR2 or iRCox-1, its effects were manifested by the inhibition of half of the expression level even one day after electroporation (the values decreased to 63.2 and 51.9% of control for GluR2 and Cox-1, $n = 4$ and 3, respectively; Fig. 2F). Three to 6 day after surgery, the levels of cDNAs encoding Cox-1 and GluR2 decreased markedly, (the values decreased to 23.2 and 17.2% of control at 6 day after electroporation for GluR2 and Cox-1, $n = 5$ and 5, respectively; Fig. 2F). However, this effect of RISLE became weaker after 6 day (the values decreased to 73.2 and 64.1% of control at 2 wk after electroporation for GluR2 and Cox-1, $n = 3$ and 4; Fig. 2E) and returned to nearly the same level as that before electroporation (the value decreased to 87.5 and 92.4% of control for GluR2 and Cox-1, $n = 3$ and 3, respectively; Fig. 2F). These indicate that the effect of RNAi is rapid and strong on mRNA expression level,
in spite of the comparatively long life spans of ubiquitous proteins such as Cox-1 and of limbic membrane-associated proteins such as GluR2. Thus the delayed effect of RNAi on mRNA expression level may be due to residual proteins the naturally occurring degeneration of which is relatively slow. However, it was found that the effect of RISLE is transient. Therefore we used the samples 5–7 day after electroporation throughout the experiments except for the time course experiments.

Specificity of siRNA

To confirm that the scrambled siRNAs used were necessary, we checked the comparison of the efficacy of mRNA depletion from individual different siRNA duplexes for the RISLE targeting visual cortex. Consequently, each siRNAs duplexes had an effect on the knockdown of the target mRNA of GluR2 or Cox-1 at ~50% (n = 3–4; Fig. 3). However, it has been elucidated that the cocktails of all the four siRNA duplexes have the strongest effect on the knockdown of the target mRNAs. These results confirm the specificity for the scrambled siRNAs used.

RISLE has no effects on expression of proteins other than the target

To determine whether the effect of RISLE is specific for the target protein, we examined the levels of proteins other than the target protein, including homologous proteins in the visual cortex after RISLE. We analyzed Cox-2, the expression of which is enhanced by long-term potentiation (LTP) in an activity-dependent manner; Cox-2 is also ubiquitously found in small amounts in the brain (Yamagata et al. 1993). Moreover, we analyzed GluR1 and NR1, both of which play important roles in LTP and long-term depression (LTD); glutamate receptor interacting protein 1 (GRIP1) and glutamate receptor interacting protein 2 (GRIP2), both of which can associate with GluR2 (Barry and Ziff 2002); and α-tubulin as a control. Consequently, we found that the expression levels of Cox-2, GluR1, NR1, GRIP1, GRIP2, or α-tubulin protein are not significantly affected by RISLE, whereas the levels of target proteins markedly decrease when RISLE is carried out using the target siRNA (Fig. 4). This suggests that the effect of RISLE is target-specific and that the decrease in the expression level of a target protein by RISLE is not accompanied by a change in the expression levels of nontarget proteins.

Range of brain region influenced by RISLE

To investigate the range of effect of RISLE, immunohistochemicals were performed 6 day after electroporation using siRNA of iRGluR2 or iRCox-1. The effects of RISLE were exclusively observed in the region to which RISLE was applied. The area where expression was attenuated was ~1–2 mm wide (Fig. 5, A, C, and D) and fully distributed between the two electrodes, the width of which was 5 mm (Fig. 5B), with a strong effect of RNAi around the siRNA injection site. In particular, the expression level of GluR2 or Cox-1 in pyramidal cells in the CA1 region of the hippocampus was exclusively reduced when RISLE was applied stereotaxically to the CA1 region of the hippocampus (Fig. 5, C and D).

RISLE does not exert significant pathological action

Because too-strong electrical pulses of electroporation induce pathological changes, we examined whether cell death occurs in the tissues subjected to RISLE. Apoptotic cells show the terminal deoxyx nucleotidyl transferase-mediated dUTP

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**FIG. 3.** Specificity of siRNAs used in RISLE. Total RNA was isolated from the visual cortex 6 day after the injection of an individual or a cocktail of 4 different iRGluR2 duplexes (iRGluR2a–d) or iRCox-1 duplexes a–d (iRCox-1a–d). Then analysis was performed as in Fig. 2F.
nick-end labeling (TUNEL)-positive change in the nucleus. When AcD, known to induce apoptosis (Hatayama et al. 2001), was injected into the visual cortex, apoptosis occurred around the injection site, although no significant apoptotic change was observed on the contralateral side of RISLE with iRGluR2 (Fig. 6A).

When an excessive amount of glutamate was applied, on the other hand, neuronal apoptosis and/or necrosis occurs (Choi et al. 1987) and PI was taken up into cells the membrane integrity of which had degenerated, followed by PI binding in the nucleus. Cell death was severe around the glutamate injection site in the visual cortex, which was almost intact on the side of RISLE with iRGluR2 (Fig. 6B). Similarly, RISLE with iRCox-1 showed no significant apoptic change or cell death as observed in AcD or glutamate treatment (data not shown).

Some studies have shown that some siRNA may induce interferon-stimulated genes in vitro (Bridge et al. 2003; Sletz et al. 2003). Therefore we measured the expression of OAS1, a classic interferon target gene. The expression levels of OAS1 in the tissue of the visual cortex subjected to RISLE with iRGluR1 or iRCox-1 were less than twofold that of OAS1 in the tissue of the visual cortex subjected only to RISLE without siRNAs (Fig. 6C). It has been reported that OAS1 is induced by >50-fold on activation of an interferon response (Bridge et al. 2003). Therefore it has been found that the siRNAs used in the current study have no significant effect on inducing interferon-stimulated gene.

Analysis for glutamate release

The subcellular location of GluR2 is limited to postsynaptic sites (Petralia and Wenthold 1992). To determine whether synapses in GluR2-knockdown rats subjected to RISLE have intact functions such as glutamate release, glutamate release from synaptoneurosomes induced by KCl or the BDNF was measured (Fig. 7A). Synaptoneurosomes on the control side showed the effects of KCl or BDNF on the release of glutamate consistent with previous papers (Jovanovic et al. 2000; Sihra et al. 1992); those from the RISLE-subjected region of GluR2-knockdown rats showed similar effects. This suggests that presynaptic functions such as glutamate release from synaptic terminals are intact even when the expression level of GluR2 is reduced.
Electrophysiological recording from tissue subjected to RISLE

We investigated whether electrophysiological phenomena can be induced in GluR2-knockdown rats by RISLE. Our previous studies have shown that in the visual cortex, LTP in vivo increases to ~120% of the baseline, and LTD in vivo is absent without the blockade of endogenous BDNF (Jiang et al. 2001, 2003). Similarly, the effects of electroporation without siRNA on LTP in the visual cortex was almost the same as those observed in the control, and LTD was not induced in the electroporation-subjected visual cortex without siRNA (Fig. 7B). Previous genetic manipulation studies showed that LTP in GluR2-knockout mice is greater than that of the wild-type and that the basic transmission of evoked field potential is greatly decreased, although LTD is not affected (Jia et al. 1996; Meng et al. 2003). In the present study, in vivo LTP in GluR2-knockdown rat increased to ~150% and the basic transmission of evoked field potential was significantly reduced (the value decreased to 58.3% of control, n = 11; Fig. 7B), whereas in vivo LTD was not affected (Fig. 7B).

To examine the effect of RISLE on electrophysiological responses in vitro, LTP and LTD were investigated using acute slices of the visual cortex transfected with or without iRGluR2 by RISLE. Previous works including ours have shown that LTP and LTD in the visual cortex in vitro increase to ~115–120% and decrease to 70–80% of the baseline, respectively (Akaneya et al. 1996, 1997). Similar to these studies in vivo, LTP in the visual cortex transfected with iRGluR2 by RISLE increased to ~150%, and the basic transmission of evoked field potential was significantly reduced (the value decreased to 43.9% of control, n = 12; Fig. 7B). Moreover, the effects of RISLE with iRGluR2 on LTD were almost the same as those of electroporation without siRNA (Fig. 7B). Also unlike the finding from GluR2-knockdown rats, the basic transmission of evoked field potential in Cox-1-knockdown rats was normal (data not shown), suggesting that the reduction in the basic transmission of evoked field potential in GluR2-knockdown rats is due to the RNAi of iRGluR2 and not to RISLE on its own. These results suggest that RISLE per se is applicable to electrophysiological experiments as confirmed by the consistency of the results between conventional KO studies and the present study.

DISCUSSION

Viral infection with siRNA is effective for RNAi in vivo (Xia et al. 2002). However, it is difficult for viral infection to regulate the extent of the region where the effect spreads and to avoid cell death. Moreover, there are several limitations in the use of viral infection: potential toxicity to neurons, neuronal protein synthesis, antibody generation, highly time consuming, high construction labor, cost, and potential hazard to laboratory personnel (Ehrengruber et al. 2001; Janson et al. 2001). Moreover, lipofection is available to RNAi (Baker-Herman et al. 2004), although this technique is less efficient to postmitotic cells and cytotoxic in some cases. On the other hand, RISLE can overcome these limitations by accommodating the region where the effect of RNAi is reached by controlling the factors such as the siRNA injection site, amount of solution, lag time of electroporation after injection of siRNA,
and site of electrode injection for electroporation and by maintaining the physiological conditions for the target region. Indeed, we have demonstrated the inhibition of apoptosis or cell death and the maintenance of the physiological functions of glutamate release from presynaptic sites, LTP, and LTD with the same results as those reported previously (Jia et al. 1996; Jovanovic et al. 2000; Meng et al. 2003; Sihra et al. 1992).

RNAi is a powerful tool for gene knockdown. However, double-stranded RNA (dsRNA) >30 nucleotides activates a dsRNA-dependent protein kinase, which leads to the activation of the type-I interferon-response, global shutdown of translation, and final marked alteration in cellular metabolism (Gil and Esteban 2000). Recent works showed that some siRNAs induce interferon-stimulated genes in mammalian cells in vitro (Bridge et al. 2003; Sletz et al. 2003). However, it remains unknown whether siRNA induces interferon response genes in vivo. The introduction of siRNAs into the rat brain in vivo did not lead to the significant activation of interferon-response genes such as OAS1. Therefore it can be said that siRNAs such as iRGluR2 and iRCox-1 can be safely introduced into the brain in vivo. To ascertain the safe introduction of other siRNA in vivo, further investigation is necessary.

The major disadvantage of electroporation is the need to increase voltage to enhance the efficiency of transfection, which may increase the possibility of inducing cell death (Itasaki et al. 1999). Strong electrical shocks increase the effectiveness of transfection by increasing the size and number of pores in the membrane but spontaneously enhance cell death. This is due to the increased toxicity following the influx of external media, generation of free radicals (Bonafous et al. 1999) and vascular effects (Gehl et al. 2002). When the lifetime of pores is prolonged by electric pulses of long duration, intracellular contents may leak out of the membrane (Gehl et al. 1999). The voltage used in previous studies is extremely higher than that in the present study (Itasaki et al. 1999). A similar technique using electroporation and siRNAs has been reported recently, although the prolonged duration of high-voltage electrical shocks used in that study may have induced pathological changes not only in the target but also in the non-target regions between which tweezer-type electrodes were placed (Matsuda and Cepko 2004). The main reason for the avoidance of pathological changes following RISLE in spite of the success of this method in a wide range of knockdown may be the use of very weak electric pulses, unlike those used in conventional methods of electroporation. In other in vivo electroporation studies, the parameters of the electrical pulse with 10- to 90-V intensity and >50-ms duration are generally used (Itasaki et al. 1999). In this study, we used a combination of the preceding electrical pulse of high intensity and short duration (poring pulse) and the following electrical pulse of low intensity and long duration (driving pulse). The two electrical pulses have their own respective task. It is presumed that the poring pulse opens the pore in the membrane, and the driving pulse leads extracellular siRNAs to enter the cytosol. The intensity of the poring pulse is remarkably higher than that of the single electrical pulse used in other studies, whereas the duration of the poring pulse is extremely shorter than that of the single electrical pulse used in other studies. The short duration of the poring pulse and the low intensity of the driving pulse decrease the strength of the electrical pulse, in spite of the high intensity and long duration of these pulses, respectively. These are effective for decreasing the damage of tissue subjected to electroporation. On the other hand, the high intensity of the poring pulse and the long duration of the driving pulse are important for the distinct roles of these pulses.

Another advantage of RISLE is that there is a limited region for the knockdown. In other in vivo and in ovo electroporation studies, electroporation is subjected systemically or to all organs such as the brain, liver, kidneys, or muscles. This may induce damage to a wide range of body organs and lead to the difficulty in limiting the target region for knockdown. The RISLE also may have some difficulty to limit existing region of siRNAs by introducing the siRNAs to the target region. However, RISLE can control the knockdown region by altering the width of each electrode in a pair. Thus the knockdown of the off-target region can be prevented by RISLE.

It is possible to accommodate the knockdown region by inserting the electrodes after the systemic administration of siRNAs. Unlike in the case of the viral vectors, however, it is difficult for siRNAs to pass the blood brain barrier to reach the target cells (Trüllsch and Wood 2004). Therefore the direct injection of siRNAs into the target region may be better than systemic administration.

The emergence and maintenance of effect of RNAi depend on species and siRNAs used. The effect of RISLE was transient and was most effective 1 wk after electroporation. This is due to the apparent lack of mechanisms for amplifying silencing unlike in the cases of worms and plants (Wall and Shi 2003). To overcome this disadvantage of RISLE, it may be effective to use several plasmid-vector systems that produce short-hairpin RNAs that are driven by promoters dependent on RNA polymerase III (Pol III) (Paddison et al. 2002; Sui et al. 2002) or Pol II (Shinagawa and Ishii 2003; Xia et al. 2002).

RISLE is expected to have experimental applications. Depending on the volume, shape and distribution of the region to be knocked down, the isolation-free length at the tip should be changed by coating or removing EPICO, and the amount of siRNA, injecting-duration delayed application of electric shocks after injecting siRNA, and stereotaxical determination of the target region should be changed. It is possible to use RISLE in establishing animal models of diseases, for example, producing a model for ischemia, Parkinson’s disease, or Alzheimer’s disease by introducing the siRNAs of related genes into a specific region. The alternative applications of reducing the expression level of a target protein, on the other hand, include the clinical treatment of diseases and gene therapy. As a phenotype for gene therapy, RISLE may be used in the therapy of diseases caused by abnormal proteins and may be available for not only small target regions but also other substantial organs such as the liver, kidneys, and muscles.

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References


